EXOPOLYSACCHARIDE BIOSYNTHESIS

IN *XANTHOMONAS* SP.

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Doctor of Philosophy

Department of Microbiology

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To Dorothea with thanks

. CONTENTS .

ABSTRACT

Xanthomonas campestris is a gram-negative, plant pathogenic bacterium which produces copious amounts of extracellular slime polysaccharide during growth. The wild-type Xanthomonas T646 produced an exopolysaccharide containing glucose, mannose and glucuronic acid in a molar ratio of 2 : 2 : 1 ; amounts of pyruvate and to a lesser extent, acetate varied with carbon source.

Two classes of mutant were isolated following mutagenic treatment of Xanthomonas T646. Stable, non-mucoid mutants produced trace quantities of exopolymer, chemically identical with the wild-type product. Crenated mutants of X. campestris produced unusual colonies on solid media; the exopolysaccharide produced by crenated strains contained material identified as X. campestris xanthan exopolysaccharide using a specific depolymerase.

Analysis of lipopolysaccharide fractions from these strains showed that the wild-type polysaccharide contained predominantly glucose. The polysaccharide fractions of the two classes of mutant were chemically similar and contained rhamnose, galactose and smaller amounts of glucose. Crenated strains were probably "rough" or "semi-rough" and observed phenotypic changes were discussed in terms of cell surface alterations.

Pathways of glucose metabolism were similar in parent and non-mucoid bacteria, but fundamentally different in the crenated strains. The possibility of control of exopolysaccharide synthesis at the metabolic level, was discussed.

Synthesis of exopolysaccharide involved solvent extractable intermediates, tentatively identified as the isoprenyl pyrophosphoryl derivatives of glucose and cellobiose. A particulate system produced similar intermediates during synthesis of variable chain length, water-soluble oligosaccharides containing $\beta 1 \rightarrow 4$ linked glucosyl residues. Synthesis of both intermediates and polymer was sensitive to bacitracin. Similarities with bacterial cellulose synthesis in Acetobacter xylinum were discussed and the possibility of a novel mechanism of polysaccharide biosynthesis was considered.

ACKNOWLEDGEMENTS

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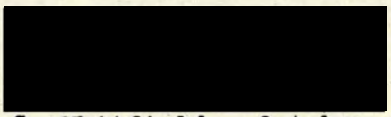
I am grateful to Dorothea and to my parents for their support during this study and particularly to my mother who mastered a new language in the typing of this thesis.

Finally, but not least, I would like to thank my colleagues in Edinburgh and Kent for their friendship and ceaseless criticism throughout this study!

DECLARATION

I hereby declare that this thesis has been composed by myself and that the research presented is, to the best of my knowledge, my own. Due acknowledgement is made within the text for any contributions received from my colleagues.



C. Whitfield October 1979

ABBREVIATIONS

Abbreviations used in this thesis were as recommended by the Biochemical Journal. In addition the following specific abbreviations were used:

Glc, Glucose ; Gal, Galactose ; Man, Mannose ; Hep, Aldoheptose ; Rha, Rhamnose ; Fuc, Fucose ; Abe, Abequose ; Rib, Ribose ; Gln, Gluconate ; $GlcNH_2$, Glucosamine ; GlcNAc, N-Acetyl Glucosamine ; Mur NAc, N-Acetyl Muramic Acid ; GlcA, Glucuronic Acid ; KDO, 2-Keto-3-Deoxyoctonic Acid ; EtN, Ethanolamine ; Ala, Alanine ; Lys, Lysine ; DAP, Diaminopimelic Acid ; Glu, Glutamic Acid ; Ac, Acetyl ; Pyr, Pyruvate ; PEP, Phosphoenolpyruvate ; P, Orthophosphate ; NH_4Ac, Ammonium Acetate ; CM, Chloroform/Methanol (2 : 1, v/v) ; CMW, Chloroform/Methanol/Water (10 : 10 : 3, v/v) ; PAAN, Peracetylated Aldonitrile Derivative ; LPS, Lipopolysaccharide

INTRODUCTION

SECTION 1 The Bacterial Cell Surface.

a) Surface layers of the bacterial cell.

A variety of polysaccharides are produced by the bacterial cell, varying in component diversity, fine structure and function. Functionally, these polymers may take the form of intracellular stores of carbon and energy; structural components such as peptidoglycan, teichoic acid and lipopolysaccharide in the cell wall, or capsular polysaccharides. Alternatively, polysaccharides may be excreted freely into the environment as loose slime. This review will concentrate upon the structure and biosynthesis of those polysaccharides which collectively and with the participation of a variety of non-carbohydrate components, comprise the bacterial cell surface (fig. 1).

The cytoplasm of both gram-negative and gram-positive bacteria is enclosed within the cytoplasmic membrane. Outwith this membrane lies the cell wall which contains features unique to either gram--positive or gram-negative bacteria and also common features such as the presence of peptidoglycan. The ability to form capsular and slime exopolysaccharides is widely found throughout bacterial genera.

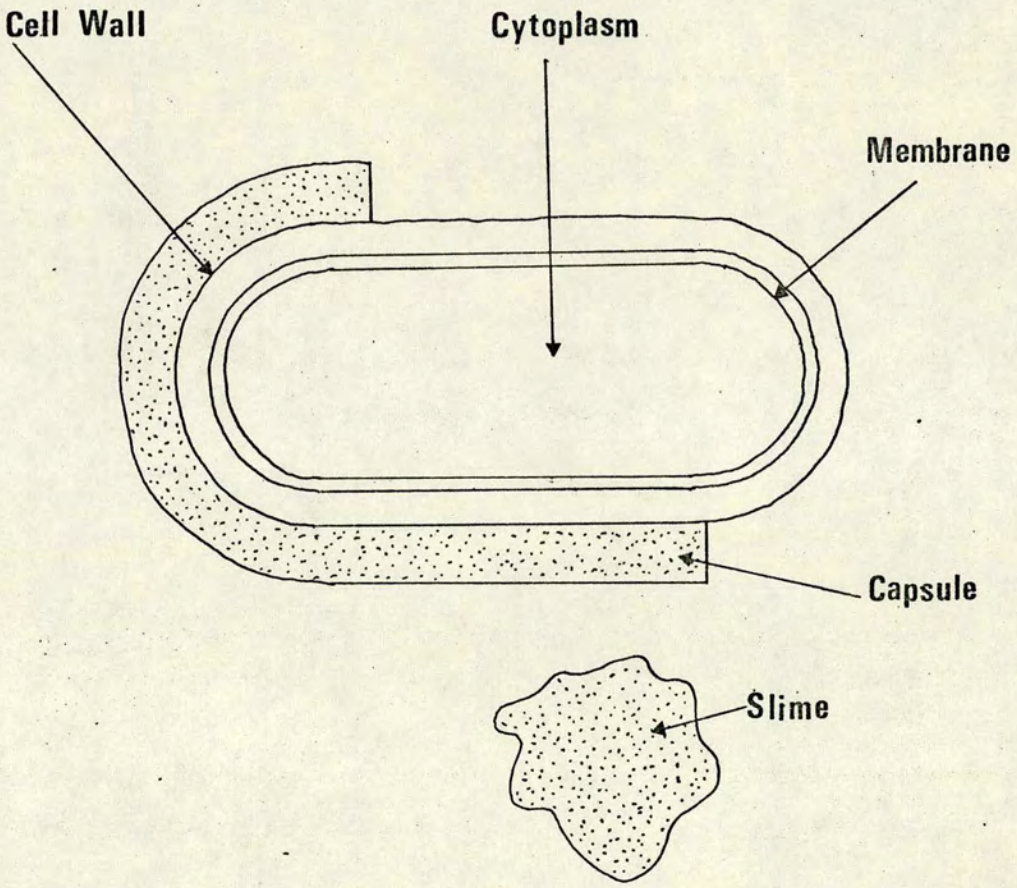
b) The gram-negative cell envelope.

(i) Cytoplasmic membrane.

The cytoplasmic components of the cell are enclosed within a cytoplasmic membrane consisting almost entirely of protein (45-70%) and lipid (10-35%). Gel electrophoresis studies reveal a variation in proteins from organism to organism, the composition is complex (eg Reaveley and Burge, 1972). The lipids of membranes are largely polar, phosphoglycerides predominate. Phosphatidylethanolamine is the predominant species in gram-negative bacteria, phosphatidylglycerol and cardiolipin in gram-positive bacteria. In gram-positive and in some gram-negative bacteria (Shaw, 1970) glycolipids have also been detected.

Structurally, the cytoplasmic membrane is seen as a lipid bilayer (Danielli and Davson, 1935). The most satisfactory model, that is the one adhering to most of the observed data, is the fluid mosaic model postulated by Singer and Nicolson (1972). The selective

Fig. 1 The bacterial cell surface



osmotic barrier provided by the hydrophobic membrane forms a vectorial matrix in which particulate (membrane-bound) enzymes may function with spatial separation of substrate and product, but also, a solid structure to which ribosomes can be attached.

(ii) Outer membrane.

Gram-negative bacteria possess a cell envelope comprising a second trilamellar membrane (outer membrane), delimiting a zone termed the periplasmic space outside the cytoplasmic membrane (fig. 2).

Above and closely associated with the inner membrane is an electron dense layer 3-8 nm thick in Escherischia coli (Murray, Steed and Elson, 1965). This layer, peptidoglycan, is synthesised at the cytoplasmic membrane and it is thought that the two structures may be joined by nascent peptidoglycan (Rogers, 1970). Close contact between the cytoplasmic membrane and the rigid peptidoglycan would be expected to result from cellular turgor pressure.

The outer membrane is seen in most studies as being an essentially smooth, unit membrane structure, 6-10 nm in thickness (Reaveley et al., 1972), however a regular pattern may be observed in some organisms eg. the hexagonal protein sub-unit layers of Spirillum serpens. Structurally, this outer membrane comprises phospholipid (26%) and protein (11%), but its uniqueness is due to the presence of lipopolysaccharide (60%).

Lipoproteins 12-14 nm long and comprised of 57 amino acids (Braun and Bosch, 1972) extend between the outer membrane and the peptidoglycan in some gram-negative bacteria (Braun and Rehn, 1969; Braun and Sieglin, 1970; Braun and Wolff, 1970). The outer membrane is suggested to be anchored by hydrophobic interactions between covalent lipid in the lipoprotein and outer membrane phospholipids (Schnaitman, 1971). This proposal is supported by the observation that protein degradation results in breakdown of the integrity of the envelope (Braun et al., 1970; de Petris, 1967; Thornley and Glauert, 1968). The inelastic foundation provided by the peptidoglycan-lipoprotein interactions (Kolenbrander and Ensign, 1968;

Fig. 2 Schematic diagram of the gram-negative cell envelope
from Costerton, Ingram & Cheng, (1974)

key to symbols:


peptidoglycan


polypeptide cross link


phospholipid


Lipopolysaccharide schematic


Lipopolysaccharide


enzymatically active protein

+ free cation — anion

⊕ bound cation ⊖ anion


covalent bond


adhesion point produced by ionic binding

bp binding protein

ec enzyme with cytoplasmic function

em cell wall biosynthetic enzyme

ep periplasmic enzyme

es enzyme located at cell surface

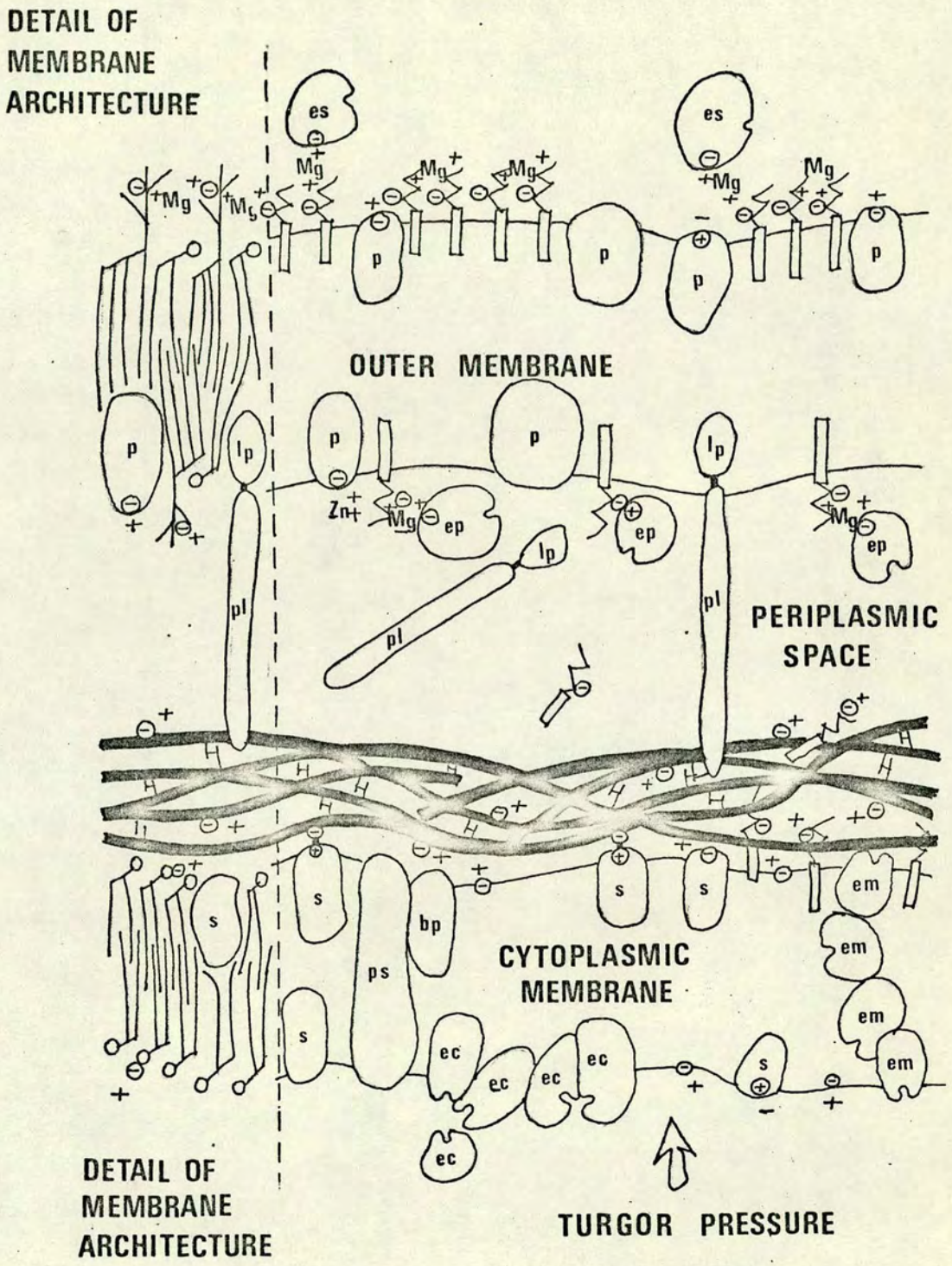
p outer membrane protein

ps permease

s structural protein

pl protein of Braun's lipoprotein

lp lipid of Braun's lipoprotein


DETAIL OF MEMBRANE ARCHITECTURE
es
Mg
p
OUTER MEMBRANE
lp
pl
Zn
ep
PERIPLASMIC SPACE
s
bp
ps
ec
em
CYTOPLASMIC MEMBRANE
DETAIL OF MEMBRANE ARCHITECTURE
TURGOR PRESSURE

Buckmire and Murray, 1970), may be the equivalent of the gram--positive cell wall.

(iii) The periplasmic space.

The periplasm (Mitchell, 1970) is a compartment bounded by the cytoplasmic and outer membranes in gram-negative bacteria and its importance lies in its enzyme content. Periplasmic enzymes, binding proteins and pigments are released into the environment when the outer membrane integrity is disturbed by techniques such as amino acid deprivation, pH increase, polymyxin treatment, or interference with either protein or peptidoglycan synthesis (Costerton, Ingram and Cheng, 1974). A similar effect is achieved by 'leaky' mutations. As a result, enzymes are often released as complexes with lipopolysaccharide eg alkaline phosphatase (Lindsay, Wheeler, Sanderson and Costerton, 1973).

Enzyme distribution within the periplasm remains open to individual interpretation. Cheng, Ingram and Costerton (1971) suggest that since it is possible to 'permeabilise' the outer membrane allowing large molecules into the cell with no apparent loss of alkaline phosphatase, this enzyme must be bound to a structural component. This is supported by release of alkaline phosphatase under certain conditions (see above) not as free enzyme, but as an enzyme-lipopolysaccharide complex (Lindsay et al., 1973). Ferritin labelling studies indicate even distribution of alkaline phosphatase (McAlister, Costerton Thompson, Thompson and Ingram, 1972), whereas other workers suggest localisation within the enlarged periplasmic space at the polar cap (Wetzel, Spicer, Dvorak and Heppel, 1970).

c) The gram-positive cell envelope.

The cytoplasmic membrane of gram-positive bacteria resembles that found in gram-negative organisms. Unlike the gram-negative envelope, that of the gram-positive cell has no second, outer membrane but only peptidoglycan and in most gram-positive organisms, teichoic acids, situated outwith the cytoplasmic membrane. Teichoic acids may be linked covalently to peptidoglycan or in the case of membrane teichoic acids, to lipid residues (see Section 5). Location of

teichoic acid in the cell wall may occur in one of two alternative ways. Either the peptidoglycan chains lie perpendicular to the surface with about 40% of these chains attached to teichoic acid in a surface layer, or glycan chains lie parallel to the surface with uniform distribution of teichoic acid throughout the wall (Archibald, Baddiley and Heckels, 1973).

d) The relationship of exopolysaccharides to the cell surface.

Many bacteria, both gram-positive and gram-negative are capable of producing polysaccharides which are located external to the cell wall as either capsule or slime material (fig. 1). Visualisation of the hydrophilic capsule by negative staining (eg India ink method of Duguid, 1951) or the Quelling reaction (Cruickshank, 1966) demonstrates a discrete layer. The use of more advanced techniques with electron microscopy (see Roth, 1977), has given more information concerning capsular localisation but since these techniques often result in dehydration, results must be regarded with caution. Bacterial slimes are excreted freely into the surrounding milieu with no apparent attachment to the cell surface.

The mode of linkage of capsular polysaccharide to the cell surface remains unclear. The attachment of capsule to lipopolysaccharide has been suggested for Enterobacter aerogenes, on the basis of contamination of exopolysaccharide preparations with sugars unique to the lipopolysaccharide (Wilkinson, Dudman and Aspinall, 1955 ; Nimmich, 1969). However similar contamination of slime exopolysaccharide preparations has also been reported and therefore, such results may only reflect loss of lipoprotein--lipopolysaccharide complexes during growth (Rothfield and Pearlman-Kothencz, 1969 ; Knox, Cullen and Work, 1967).

The definition of transition from capsular material to slime is arbitrary and mutations from capsule to slime production are relatively common. Differences between the two forms of exopolymer from a given strain are slight, the polysaccharides being of similar chemical composition where characterised (Wilkinson et al., 1955 ; Aspinall, Jamieson and Wilkinson, 1956). Mutations in

lipopolysaccharide biosynthesis may cause alterations in surface protein composition (see Ames, Spudich and Nikaido, 1974 ; Kaplow and Goldfine, 1974). Such changes may alter possible sites of capsule binding, causing the release of slime exopolysaccharide.

SECTION 2 Precursors for Polysaccharide Biosynthesis :

Nucleotide Diphosphate Sugars.

a) Structure and function.

In an early study of lactose metabolism in Saccharomyces fragilis (Caputto, Leloir, Trucco, Cardini and Paladini, 1948 ; Leloir, Trucco, Cardini, Paladini and Caputto, 1948), evidence was presented to indicate the central role played by sugar nucleotides in carbohydrate metabolism. The novel compound uridine diphosphate glucose was isolated and its structure elucidated (fig. 3). Subsequent research established the role of sugar nucleotides as glycosyl donors in the biosynthesis of several oligosaccharides and of glycogen (Leloir and Cardini, 1957).

The involvement of sugar nucleotides in the synthesis of cell surface polysaccharides was demonstrated initially by Smith, Mills and Bernheimer (1961), in a study of the biosynthesis of capsular polysaccharide by pneumococci. This was soon followed by illustrations of UDP-sugars as glycosyl donors in lipopolysaccharide synthesis (eg Nikaido, 1962 ; Osborn, Rosen, Rothfield and Horecker, 1962). The involvement of UDP-N-acetyl glucosamine and UDP-N-acetyl muramyl pentapeptide in peptidoglycan biosynthesis was demonstrated in 1964 by two independent groups (Chatterjee and Park, 1964, Meadow, Anderson and Strominger, 1964). Similarly, the synthesis of teichoic acids from the precursors CDP-glycerol and CDP-ribitol was also shown (Burger and Glaser, 1964).

The interconversion of sugar nucleotides allows the synthesis of a wide range of precursors from a relatively limited range of potential substrates. Such reactions have been reviewed elsewhere.

In the synthesis of complex saccharides, the glycosyl moiety is transferred from the sugar nucleotide precursor to the appropriate acceptor. Energy released on hydrolysis of the nucleotide-phosphate bond makes the formation of a glycosidic linkage energetically favourable (Leloir, Cardini and Cabib, 1960). Several systems have been shown to require PRIMERS, compounds to which glycosyl residues are added, for example cellodextrins in the synthesis of bacterial

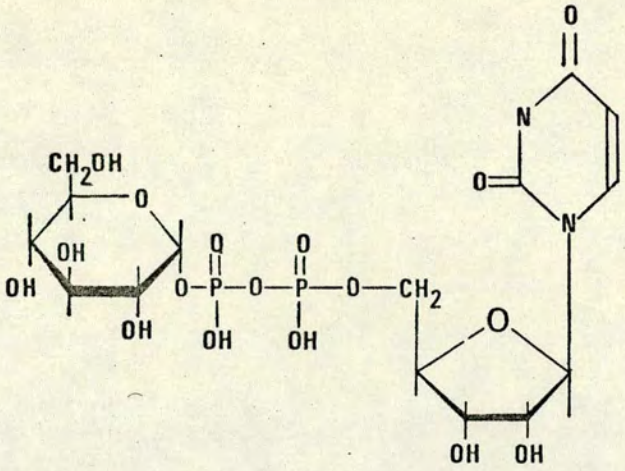


Fig. 3 Structure of uridine-5′-diphosphate glucose (UDP-glucose)

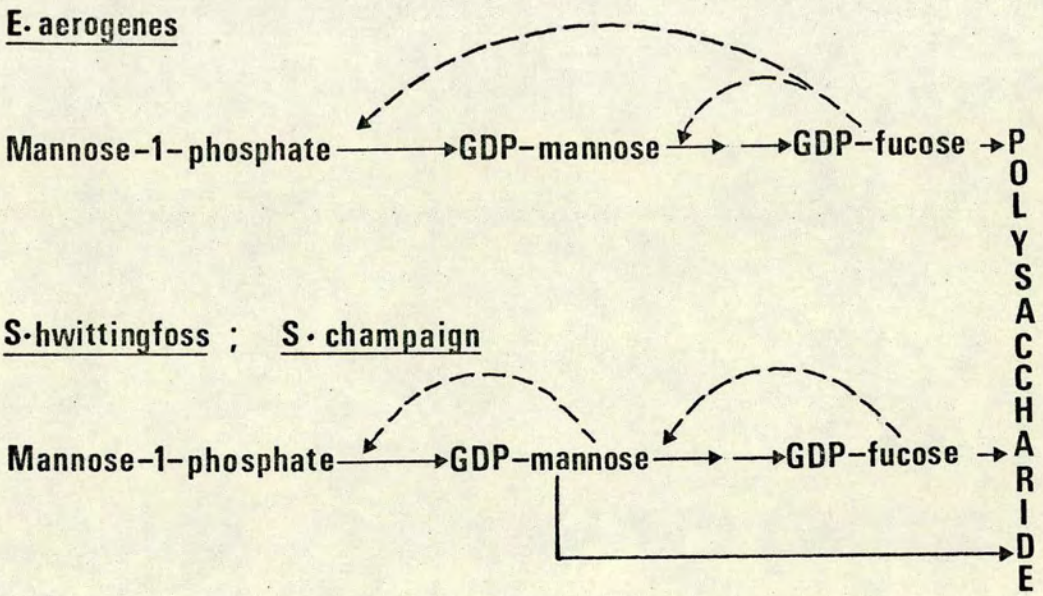


Fig. 4 Regulation of sugar nucleotide precursors involved in the biosynthesis of fucose and mannose containing polysaccharides

cellulose by Acetobacter xylinum (Cooper and Manley, 1975 b) and similarly, maltodextrins in glycogen biosynthesis (Goldemberg, 1962). Additionally, ACTIVATORS, compounds which are not incorporated into the growing chain, may be required. The involvement of glycolytic intermediates as activators in glycogen synthesis is well documented (Dietzler, Leckie, Lais and Magnani, 1974).

b) Sugar nucleotides and the control of polysaccharide biosynthesis.

Specificity of the enzymes catalysing the transfer of glycosyl residues from precursor to acceptor, GLYCOSYL TRANSFERASES, is an important factor in the biosynthesis of oligosaccharides. Three areas of specificity should be considered:-

(i) Specificity towards acceptor; In many systems, the precise nature of the acceptor and consequently the degree of acceptor specificity, remain unclear. Indirect evidence from the distribution of D-glucosyl residues in bacteriophage DNA (Kornberg, 1962) tends to suggest high acceptor specificity for these phage induced glucosylating enzymes.

(ii) Specificity towards carrier; mutants of E.coli lacking the enzyme UDP-D-glucose pyrophosphorylase (Fukasawa, Jokura and Kurashi, 1962; Sundarajan, Rapin and Kalchar, 1962) produce an incomplete lipopolysaccharide, devoid of glucose. Amounts of dTDP-D-glucose pyrophosphorylase are similar in wild type and mutant strains, suggesting a distinction between the relative abilities of dTDP-D-glucose and UDP-D-glucose to serve as glucosyl donors for lipopolysaccharide synthesis.

(iii) Specificity towards the transferred sugar; In vitro studies have demonstrated the incorporation of D-galacturonic acid but not D-glucuronic acid, from their respective UDP derivatives into pneumococcal type I polysaccharide. Alternatively, type III pneumococci incorporate D-glucuronic acid, but not D-galacturonic acid (Mills, 1960; Mills and Smith, 1962).

It is therefore apparent that metabolism and subsequent transfer of sugars in the synthesis of oligosaccharides, affords the cell a possible mechanism for the control of the biosynthetic pathway. For example, the ability of an organism to use more than

one nucleotide carrier for a single sugar may be particularly important. In strains which produce a polysaccharide containing both glucose and the 6-deoxyhexose, rhamnose, synthesis of the rhamnosyl nucleotide is thought to occur via dTDP-D-glucose ensuring continued availability of UDP-glucose for other functions. However dTDP-D-glucose has not been detected from bacterial sources although accumulation of a possible intermediate between dTDP-D-glucose and dTDP-L-rhamnose, dTDP-4-Keto-6-deoxy-D-glucose has been demonstrated in E.coli Y10 (Okazaki, Okazaki, Strominger and Michelson, 1962). It is conceivable however, that dTDP-L-rhamnose in E.coli Y10 results from non-specific reduction of dTDP-4-Keto-6-deoxy-D-glucose, during isolation and purification procedures.

In prokaryotic organisms synthesising glycogen, ADP-D-glucose serves as glucosyl donor (Greenberg and Preiss, 1964; Sigal, Cattaneo and Segel, 1964), thus functionally separating glycogen synthesis from that of cell surface polysaccharides, in which UDP-D-glucose is the principal glucosyl donor. However, since glycogen synthesis is restricted to stationary phase cells in most species studied, little synthesis of surface polysaccharides (other than turnover) would be expected.

Control of polysaccharide biosynthesis at the sugar nucleotide level occurs in E.aerogenes. The capsular polysaccharide of E. aerogenes contains either fucose or mannose; these monosaccharides are not usually found together in the polymer from a given strain. Control over fucose synthesis results from feedback inhibition of the enzymes GDP-mannose oxidoreductase and GDP-mannose pyrophosphorylase, exerted by GDP-fucose and GDP-mannose, respectively (fig. 4). However, the recent discovery of an E. aerogenes (E. ozaenae) type 6 polysaccharide containing both mannose and fucose (Elsässer-Beile, Friebolin, and Stirm, 1978), tends to contradict this generalisation. In Salmonella hwittingfoss and Salmonella champaign, the lipo--polysaccharide contains both fucose and mannose; control may be exerted at the same level (fig. 4).

Loss or change of specific enzymes concerned with the synthesis and subsequent transfer of sugars may render that organism incapable of incorporating a particular sugar into polysaccharide. Such mutations have proved an excellent tool in studies of the structure and biosynthesis of some complex polysaccharides, particularly lipopolysaccharide(see Section 6). However in the case of teichoic acids and of exopolysaccharides, these techniques have proved of little value.

SECTION 3. The Involvement of Lipids in Polysaccharide Biosynthesis.

Two classes of lipid, with vastly differing functions, are known to be involved in the synthesis of polysaccharides. A unique group of compounds the polyisoprenoid alcohol phosphates act as glycosyl carriers in many biological systems. The second, less documented role, involves the participation of phospholipids which may serve as specific cofactors required for enzyme activity.

a) Isoprenoid lipids.

(i) Structure and function.

In an idealised biological system catalysing the biosynthesis of surface polysaccharides, glycosyl donors are made available to the membrane-bound enzyme systems with a final product appearing externally. The processes which occur between these two events remain to a large extent unknown. The suggested role for the polyisoprenoid lipids is in the entrapment of sugar molecules within the hydrophobic membrane and, in conjunction with the appropriate enzymes, assisting the transfer of the sugar across the membrane (fig. 5).

Involvement of lipid-linked intermediates in the synthesis of bacterial polysaccharides was initially recognised in 1965 (Anderson, Matsuhashi, Haskin and Strominger, 1965). Analogies with other polysaccharide synthesising systems subsequently led to the discovery of similar intermediates in lipopolysaccharide O-antigen biosynthesis in Salmonella sp. (Weiner, Higuchi, Rothfield, Saltmarsh-Andrew, Osborn and Horecker, 1965; Wright, Dankert and Robbins, 1965) and later in the synthesis of E. aerogenes capsular polysaccharide (Troy and Heath, 1968; Sutherland and Norval, 1970; Troy, Frerman and Heath, 1971). The lipid involved in all these systems has been identified as undecaprenol (fig. 6). Similar compounds are now known to function in the biosynthesis of both prokaryotic and eukaryotic polysaccharides and more recently their role in eukaryotic glycoprotein glycosylation has been the subject of much investigation (see table 1)

Polyprenols are present in small amounts, accounting for a very small proportion of total membrane lipid. Quoted values vary with

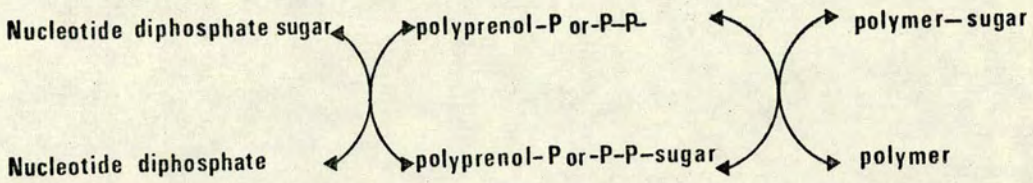


Fig. 5 Generalised function of polyprenol carrier lipids

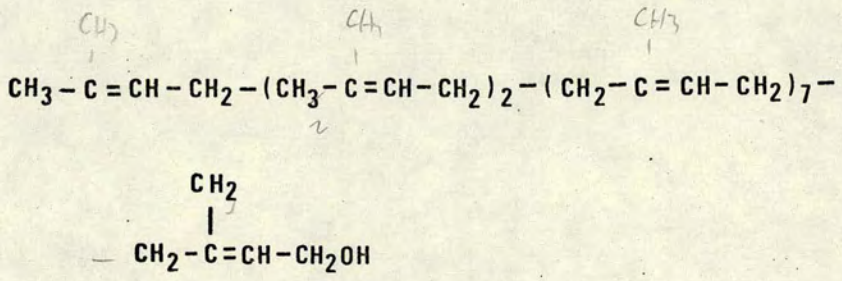


Fig. 6 Structure of undecaprenol

TABLE 1. The Involvement of Isoprenoid Lipids in Polysaccharide and Glycoprotein Biosynthesis. Adapted from Poxton (1974).

NUMBER OF ISOPRENES	TRIVIAL NAME	PRODUCT	SOURCE	LINKAGE	REFERENCE
10	decaprenol	Mannolipid	Mycobacterium tuberculosis	Phosphodiester	1
11	undecaprenol	Peptidoglycan	S. aureus	Pyrophosphate	2,3
		LPS O-antigen	S. typhimurium S. newington	Pyrophosphate	4,5,6
		O-antigen modification	S. anatum (E) also groups A,B & D	Phosphodiester	7,8
		Exopolysaccharide	E. aerogenes	Pyrophosphate	9,10
		Colanic acid	E. coli	Pyrophosphate	11
		Colominic acid	E. coli	Phosphodiester	12
	suggested undecaprenol	Cellulose	A. xylinum	Pyrophosphate Phosphodiester	13,14 15
		Mannan	M. lysodeikticus	Phosphodiester	16,17,18
		Teichoic acid	S. lactis strain 13 & 2102	suggested pyrophosphate	19,20,21
			B. licheniformis	Phosphodiester	22

Table 1 Continued

12-16	dolichols	Mammalian Glycoproteins	Mammalian Cells	Phosphodiester	23,24,25
		Algal Compounds	*Codium fragile*	?	26
14-18	dolichols	Yeast Mannan	*S. cerevisiae*	Phosphodiester	27
		Fungal Mannans	*Aspergillus* sp	Phosphodiester	28
15-16	dolichols	Yeast Glycoproteins	*S. cerevisiae*	Phosphodiester	29
	uncertain	Plant Glycoproteins	*Phaseolus aureus* *Pisium sativum*	Phosphodiester	30,31

strain and phase of growth (see Sutherland 1977b) and summarising these values, Wright (1971) has suggested a figure of 10^5 molecules of isoprenoid lipid per bacterium.

(ii) Regulation and metabolism of polyprenols.

The biosynthesis of isoprenoid lipids has been reported in several *in vitro* systems; *Micrococcus lysodeikticus* has been the source of several particulate preparations. One such system was shown to be capable of adding isopentenyl residues to a growing chain (Kandutsch, Paulus, Levin and Bloch, 1964). A product comprising 4 isoprene residues, geranyl-geranyl phosphate, was synthesised from farnesyl pyrophosphate and also from dimethylallyl and geranyl-pyrophosphates. The suggested lack of substrate specificity may indicate the presence of more than one enzyme in the unpurified preparation.

Products of a similar preparation were shown to be chains of 7 and 8 isoprene residues (Allen, Ashworth, Macrae and Bloch, 1967). The product was resistant to alkaline phosphatase activity, possibly indicating a close association between the enzyme and its growing product.

Synthesis of authentic C_{55}-undecaprenol (fig. 6) and shorter chain forms have also been reported in similar preparations of *M. lysodeikticus* (Kurokawa, Ogura and Seto, 1971). The crude preparation was thought to contain an active phosphatase since the monophosphate derivative was synthesised.

The high molecular weight product synthesised from Δ^3 isopentenyl pyrophosphate by particulate preparations of *Salmonella newington* was identical to, and exchangeable with, authentic carrier lipid in O-antigen biosynthesis. A shorter chain product was synthesised by the corresponding soluble preparation (Christenson, Gross and Robbins, 1969). C_{55}-isoprenol was also synthesised from farnesyl pyrophosphate by preparations from *Lactobacillus plantarum* (Keenan and Allen, 1974).

In a system actively synthesising polysaccharide, regeneration of a functional monophosphate derivative of the lipid from its diphosphate form (fig. 5) requires the action of a specific phos-phatase. The C_{55}-isoprenol pyrophosphatase from *M. lysodeikticus*

has been solubilised from the membrane, using the non-ionic detergent Triton X-100 (Siewart and Strominger, 1967).

The dephosphorylation of lipid diphosphate has been shown to be the principal site of action of the antibiotic bacitracin. By binding to undecaprenol diphosphate and also to shorter chain forms, the antibiotic protects the diphosphate group from the dephos--phorylating enzyme. Binding is dependent upon divalent cations and is reversed in the presence of EDTA (Siewart et al., 1967). Further dephosphorylation of lipid monophosphate to release free polyprenol is not sensitive to bacitracin (Willoughby, Higashi and Strominger, 1972).

Phosphorylation of free C_{55}-isoprenol and also of free ficaprenol (extracted from the leaves of the rubber plant Ficus elasticus), is catalysed by C_{55}-isoprenoid alcohol phosphokinase. This enzyme has been solubilised from the membranes of Staphylococcus aureus (Higashi, Siewart and Strominger, 1970) and E. aerogenes (Poxton, Lomax and Sutherland, 1974) using acid butanol (pH 4.2) extraction.

The enzyme from S. aureus has been purified (Sandermann and Strominger, 1971; 1972) and has been shown to require ATP and either phosphatidylglycerol, cardiolipin or a detergent for its activity (Higashi and Strominger, 1970) ie the second role for lipids.

The unified activity of these enzymes is capable of mediating control over the availability of polyprenol in its functional form. The kinase makes the phosphorylated polyprenol available to the biosynthetic system and the phosphatase regenerates spent lipid or, through further dephosphorylation, removes lipid from the system. Thus several schemes of regulation have been proposed (Sandermann et al., 1972; Willoughby et al., 1972) (fig. 7)

(iii) Structural polyprenols.

Although phosphorylated polyprenols are normally located within the cytoplasmic membrane, undecaprenols have been detected in mesosomal and plasma membranes of Lactobacillus plantarum (Hemming, 1974). Bactoprenols have been detected in mesosomal and plasma membranes from other lactobacilli (Thorne and Barker, 1972), the polyprenol is a C_{55}-isoprenoid alcohol with one saturated isoprene

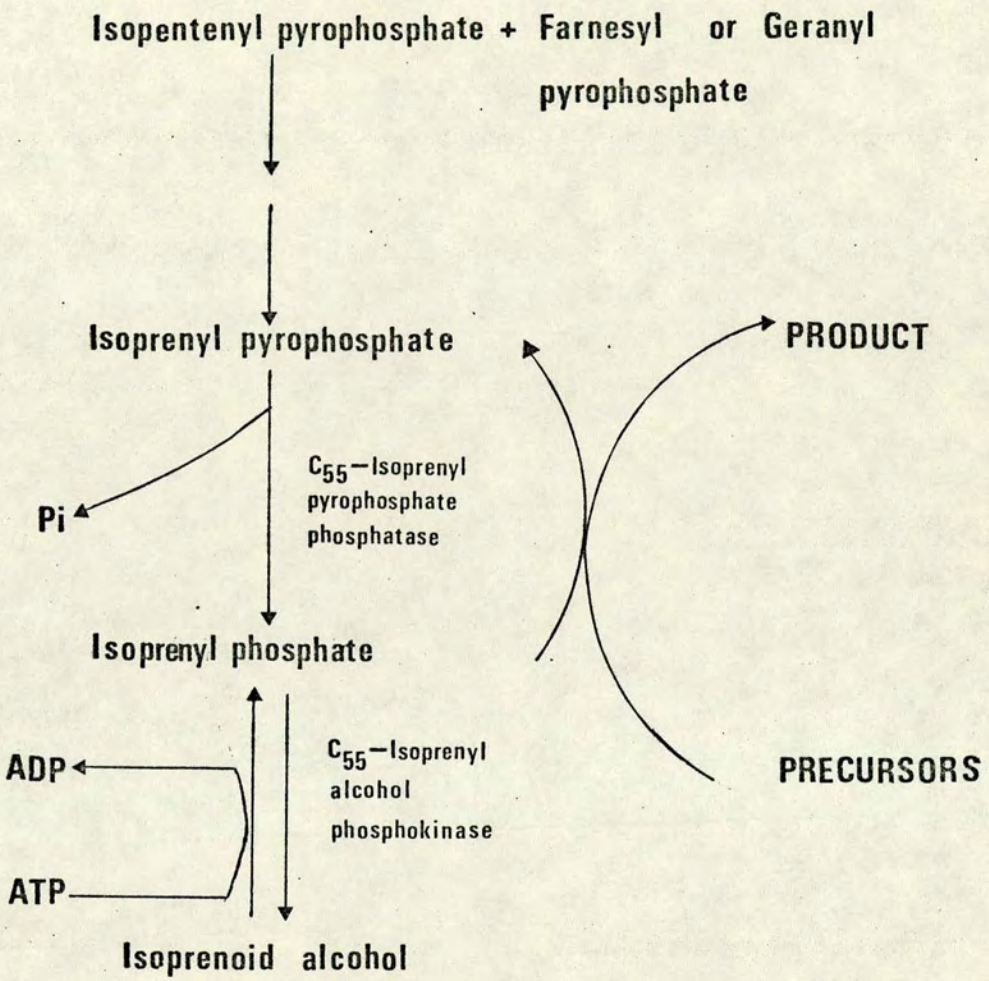


Fig. 7. Regulation and metabolism of carrier lipid from Sutherland, (1977a)

unit (Thorne and Kodiek, 1966). The polyprenol from each source has been shown to be synthesised independently.

Since many polyprenols from plant, fungal and mammalian sources are esterified to long chain fatty acids, it is unlikely that these extremely hydrophobic compounds fulfil any but a structural role.

b) Phospholipid cofactors.

As already described, C_{55}-isoprenoid alcohol phosphokinase has an absolute requirement for phosphatidyl glycerol, cardiolipin or the detergent Triton X-100. In vitro studies with several enzyme systems have indicated that reactivation to maximum activity may occasionally be favoured by addition of either phospholipid or detergent. These alternatives probably function in the same manner, by acting as surfactants; addition of both phospholipid and detergent might create mixed micelles, possibly creating an optimal environment for enzyme activity.

In vitro transfer of galactose residues from UDP-D-galactose to rat liver microsomal proteins requires the presence of CDP-choline and Triton X-100. These cofactors have been shown to exert a synergistic effect on activity (Mookerjea, Cole and Chow, 1972). Transfer of N-acetyl glucosamine in the same system also required CDP-choline (Mookerjea and Chow, 1970) but CDP-choline was not effective in stimulating mannosyl transfer in mouse myeloma systems (Hemming, 1974).

Additionally, membrane phospholipids may create changes in membrane protein conformation resulting in altered enzyme activity. This phenomenon is thought to be the causal factor in stimulation of UDP-glucuronic acid : p-nitrophenol glucuronosyl transferase, isolated from rat liver microsomes (Vessey and Zakim, 1972). In this particular system, the cofactors phospholipase A and C have been implicated.

Enzymes which are known to show such requirements, together with their cofactor requirement are summarised in table 2.

TABLE 2. Membrane Bound Enzymes With Requirements for Phospholipid or Detergent Cofactors.

ENZYME	COFACTOR
Isoprenoid alcohol phosphokinase	PG, CL, LPG, LCL
Pyruvate oxidase	LPE, PE, LPC, PC, FA & TG
UDP-Gal : LPS Gal transferase	PE
UDP-Glc : LPS Glc transferase I	CL
FAD-dependent malate dehydrogenase	CL
ATPase pyrophosphatase	PC, PS, PE
PEP-dependent phosphotransferase enzyme II	PG
α-methylglucoside phosphotransferase	PG, PS, CL (plus Mg^{++} or Ca^{++})
β-methylglucoside phosphatransferase	Anionic phospholipid or non-ionic detergent Tween 40 or Triton X-100
Phospho N-acetyl muramic acid pentapeptide translocase	Triton X-100
CDP diglyceride translocase	Triton X-100
Rat liver Gal transferase	CDP choline, Triton X-100
Rat liver N-acetyl $GlcNH_2$ transferase	CDP choline, Triton X-100
UDP-GlcA : p-nitrophenyl glucuronyl transferase	Phospholipase A and C

Table 2. (Continued)

Succinate-O_2, cytochrome c, coenzyme Q	CL, PE, PC, mixed lipids
Coenzyme Q-cytochrome c	CL, PE, PC, mixed lipids
Cytochrome oxidase	PE, CL, PC, mixed lipids
NADH-O_2, cytochrome c, coenzyme Q	PC, mixed lipids
β-Hydroxybutyrate dehydrogenase	PC
NADH-O_2, cytochrome c	PC + LPC, mixed lipids
NADPH-dependent hydroxylations	Mixed lipids
Phosphatidate phosphatase	Mixed lipids
Stearoyldesaturase	Mixed lipids + TG + FA, PC
GTP-dependent acyl-CoA synthetase	PC

Abbreviations used: CL. cardiolipin; FA, fatty acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TG, triglyceride; GPC, glycerophosphorylcholine.

Adapted from Machtiger and Fox, (1973) and Coleman, (1973).

SECTION 4 Peptidoglycan.

a) Structure.

Rigidity of the bacterial cell is a property conferred by the net action of various cell surface layers. However the major contributory factor in maintenance of shape and rigidity is the peptidoglycan layer.

Peptidoglycan is comprised of glycan strands containing peptide sub-units which are cross-linked to a variable extent. The glycan strand is essentially similar in all bacteria studied and comprises alternating $\beta 1 \rightarrow 4$ linked N-acetyl glucosamine and N-acetyl muramic acid residues. Slight variations may arise for example, as a result of variable degrees of acetylation or glycosylation of muramic acid residues in Staphylococcus aureus and Mycobacterium smegmatis, respectively (Tipper, Ghuysen and Strominger, 1965). The glycan chain length is variable, figures for S. aureus suggest 12-90 hexosamine residues, with no apparent correlation between chain length and cell shape.

Addition of peptide sub-units to the muramic acid residue is variable in both degree and composition of the peptides. Usually four amino acids are linked to the lactic acid group of muramic acid. However tripeptides have been occasionally reported, probably due to the activity of D-carboxypeptidases (Hungerer and Tipper, 1969). Alternatively pentapeptides, terminating in a non cross-linked D-alanyl residue have also been reported. A variable degree of cross-linking between adjacent peptide chains occurs; in many gram--negative bacteria, peptidoglycan is thought to be a relatively open structure due to the paucity of cross-linkages (Rogers, 1970).

b) Biosynthesis.

Although precursors of peptidoglycan, the UDP derivatives of N-acetyl glucosamine and N-acetyl muramic acid were isolated by Park and his colleagues as early as 1949, their significance in peptido--glycan synthesis remained unconfirmed until much later (Park and Strominger, 1957; Chatterjee et al., 1964). Shortly after came the significant discovery that glycolipids served as obligatory inter--mediates in the biosynthesis of peptidoglycan (Anderson et al., 1965) in both S. aureus and M. lysodeikticus. The lipid intermediate

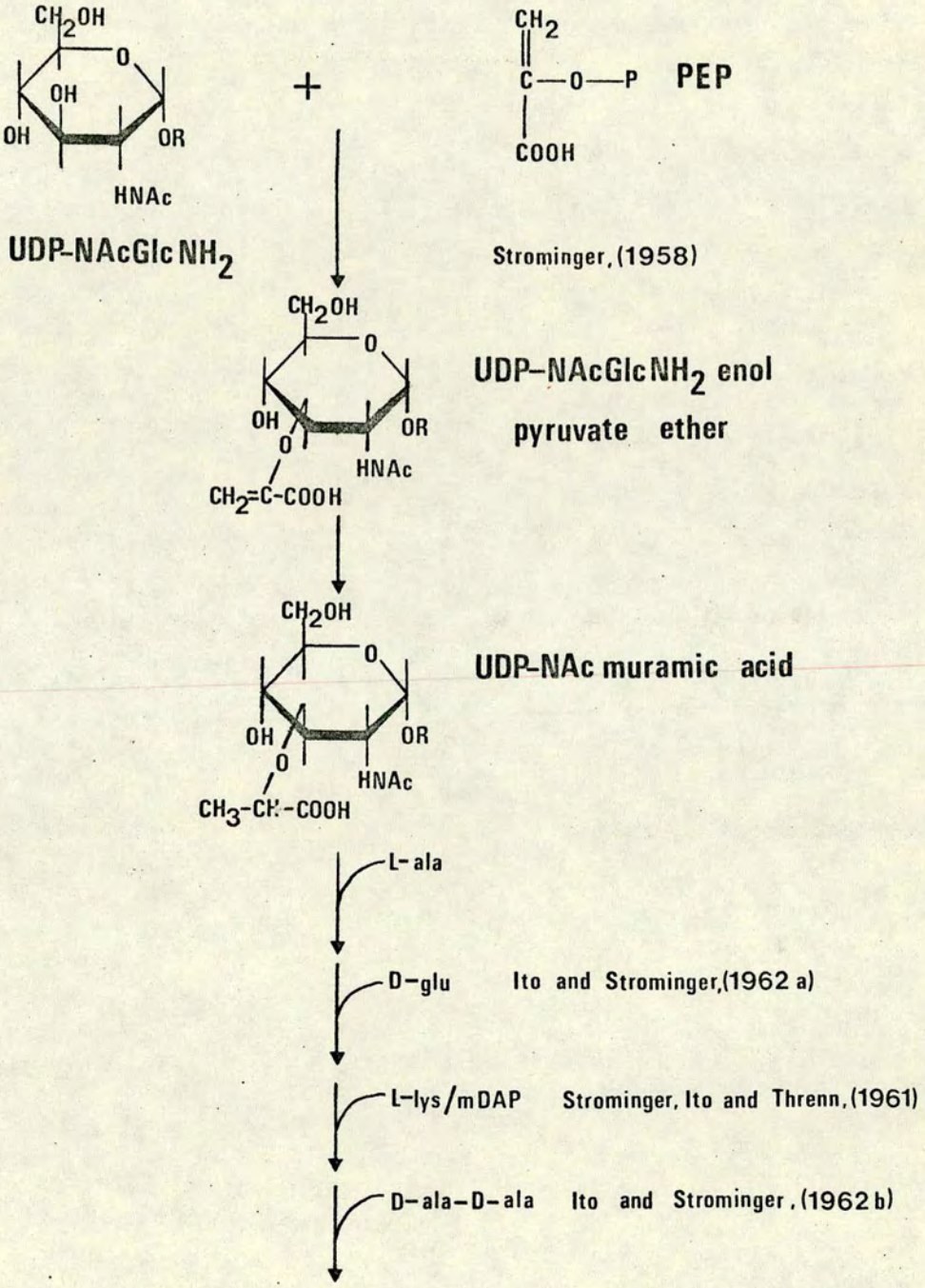


Fig. 8 Synthesis of UDP-N-acetyl muramic acid pentapeptide

was shown to contain N-acetyl glucosamine and N-acetyl muramic acids. The lipid released by acid hydrolysis of the intermediate was analysed by mass spectrometry and shown to be a polyisoprenoid alcohol containing 11 isoprene units, undecaprenol (Higashi, Strominger and Sweeley, 1967). The reducing terminus of the glycosyl residue was shown to be linked to the lipid moiety via a pyrophosphate linkage.

The majority of reports on the structure of peptidoglycan have featured gram-positive organisms and perhaps as a consequence, biosynthetic studies have been largely confined to the same group.

The initial step in biosynthesis, precursor formation, does not require the involvement of lipid intermediates. The UDP-N-acetyl muramic acid residue is formed via a lactyl ether form resulting from a condensation reaction between UDP-N-acetyl glucosamine and phosphoenolpyruvate (Strominger, 1958). The peptide chain is formed by the sequential addition of amino acids from their respective tRNA derivatives to UDP-N-acetyl glucosamine enol pyruvate ether (fig. 8).

Incorporation of cytoplasmically synthesised precursors into lipid-linked material and subsequently into peptidoglycan, is catalysed by enzymes whose activity has been demonstrated in cell wall fragments from S. aureus. Furthermore, endogenous peptidoglycan in such preparations serves as acceptor (Chatterjee et al., 1964; Meadow et al., 1964). Net synthesis of peptidoglycan results in the release of UDP and UMP. Ly specific isotopic labelling, UMP has been shown to derive from UDP-N-acetyl muramyl pentapeptideand UDP from UDP-N-acetyl glucosamine (Anderson et al., 1965).

The lipid soluble acceptor, lipid phosphate (Anderson et al., 1965) is synthesised at the membrane (Anderson, Matsuhashi, Haskin and Strominger, 1967) with N-acetyl glucosamine being incorporated directly from its UDP-derivative into preformed lipid diphosphate N-acetyl muramyl pentapeptide (fig. 9).

Modification of the peptide chain eg addition of glycine units in S. aureus, may occur at the lipid-linked disaccharide stage (Matsuhashi, Dietrich and Strominger, 1965).

Transfer of the fully modified disaccharide unit to endogenous acceptor results in net elongation of the peptidoglycan backbone and concomittant release of lipid pyrophosphate. Bacitracin sensitive

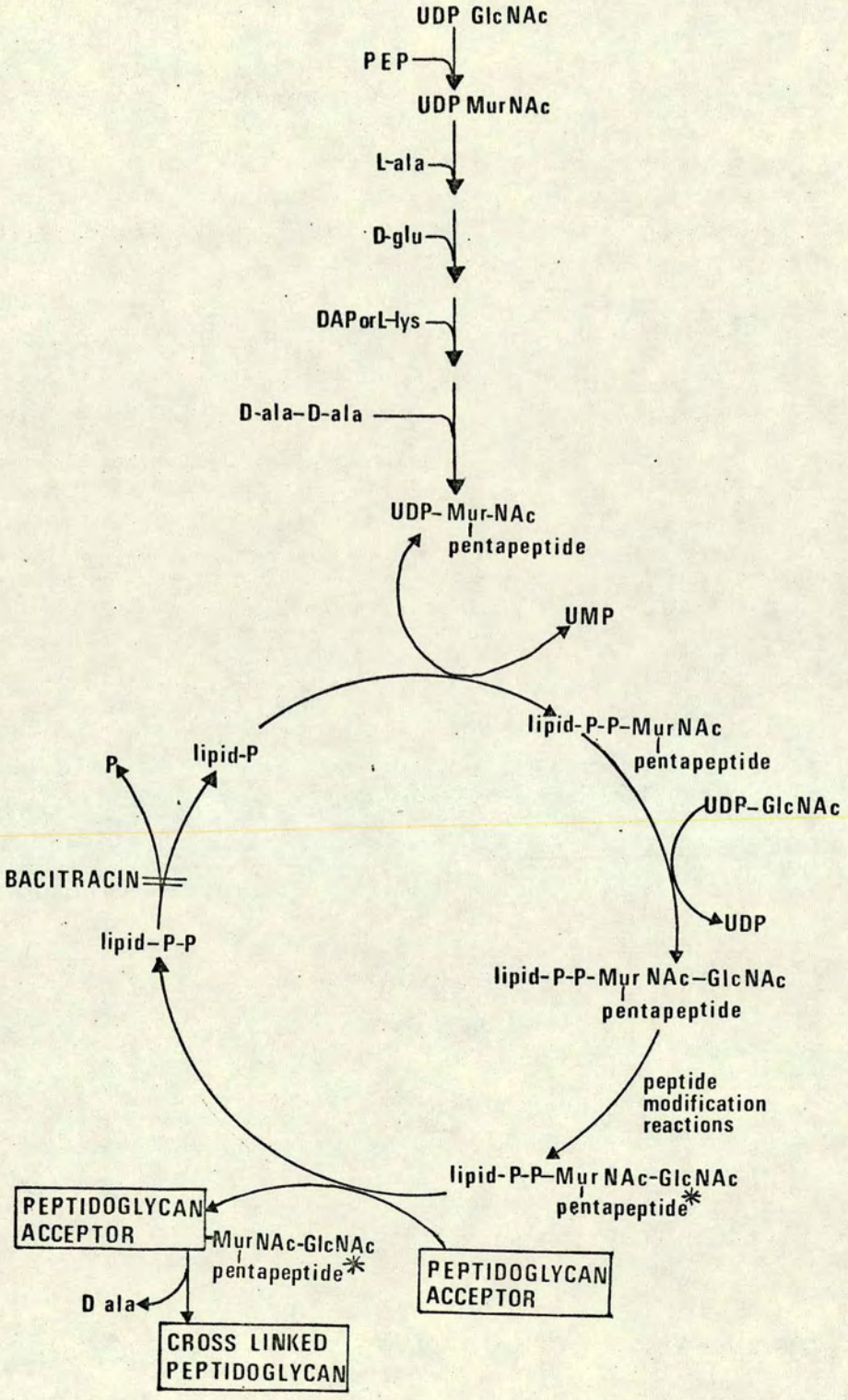


Fig. 9 Peptidoglycan biosynthesis

dephosphorylation of the released lipid derivative yields the functional monophosphate form.

Cross-linking of adjacent peptide chains is mediated by trans--peptidation reactions and with the subsequent release of D-alanine (Izaki, Matsuhashi and Strominger, 1966). This step is of particular importance since it represents the primary site of penicillin action.

The biosynthetic process may be regarded as being a cycle with lipid phosphate mediating the transfer of disaccharide repeating units to peptidoglycan acceptor, resulting in a net elongation of the nascent glycan chain (fig. 9).

SECTION 5 Teichoic Acids.

Teichoic acids are a group of related polysaccharides associated with cell wall, membrane and capsule. These polymers are found in virtually all gram-positive organisms and also in the gram-negative bacterium Butyrivibrio fibriosolvens (Sharpe, Brock, Wicken and Knox, 1975). Two broad classes of teichoic acid are recognised, cell wall teichoic acids and those associated with the membrane, lipoteichoic acids.

a) Structure.

(i) Cell wall teichoic acids.

Cell wall teichoic acids may variably comprise 30-50% of the dry weight of the cell wall. Structural studies on these readily extractable polymers reveal either polyribitol or polyglycerol chains containing phosphate groups; these structures are used to define teichoic acids. Glycerol and ribitol residues are often substituted by glycosyl or D-alanyl residues and similar compounds with phosphate and glucosamine substitutions have also been recorded. In recent years, Baddiley and his co-workers have carried out extensive research into the structure and synthesis of these compounds; their work has been reviewed by Baddiley (1972), Archibald (1974) and more recently by Duckworth (1977).

Teichoic acids are also capable of phenotypic variation : under phosphate limitation in chemostat culture, teichoic acids are replaced by teichuronic acids (Ellwood and Tempest, 1969). Teichuronic acids unlike teichoic acids show the same structure from differing sources, repeating polymers of glucuronic acid and N-acetyl galactosamine (Wright and Heckels, 1975).

(ii) Lipoteichoic acids.

Membrane or lipoteichoic acids have been studied in a number of gram-positive bacteria. Structurally these polymers have, without exception, been shown to be of the glycerol phosphate type. In Streptococcus faecalis NCIB 8191 (Ganfield and Pieringer, 1975), a teichoic acid consisting of 28-35 glycerol phosphate units partially substituted with kojibiosyl residues is linked to a lipid fraction. The lipid has been characterised as either a kojibiosyl diglyceride or more probably a phosphatidyl-kojibiosyl-diglyceride residue.

(Toon, Brown and Baddiley, 1972).

b) Biosynthesis.

(i) Cell wall teichoic acids.

The synthesis of polyribitol phosphate from CDP-ribitol was initially detected in preparations of L. plantarum (Glaser, 1963 a;b). Subsequently, the production of a polymer comprising of up to 30 glycerol phosphate residues from CDP-glycerol, was reported in preparations of Bacillus subtilis and Bacillus licheniformis. The involvement of lipid intermediates in the biosynthesis of teichoic acids has only been shown indirectly, principally by demonstrating competition for available lipid in systems where both teichoic acid and peptidoglycan are being synthesised (Douglas and Baddiley, 1968; Watkinson, Hussey and Baddiley, 1971; Anderson, Hussey and Baddiley, 1972).

The precise mechanism for the incorporation of D-alanyl residues is, as yet, unverified although an enzyme capable of complexing with ATP and alanine to form an alanyl-AMP complex has been reported. This enzyme did not however function in vitro (Baddiley and Neuhaus, 1960).

Incorporation of α- and β-linked N-acetyl glucosamine into teichoic acid from S. aureus has been reported (Baddiley, Buchanan, RajBhandary and Sanderson, 1962; Baddiley, Buchanan, Martin and RajBhandary, 1962; Sanderson, Strominger and Nathenson, 1962). Particulate preparations of S. aureus catalysed the incorporation of N-acetyl glucosamine from its UDP-derivative into a teichoic acid acceptor, depleted in N-acetyl glucosamine. Alpha and β-linkages were formed in ratios similar to the in vivo product. Intact teichoic acid could not serve as acceptor (Nathenson and Strominger, 1962; 1963).

Transfer of glucose residues to incompletely glucosylated polyglycerol teichoic acid has been reported in particulate prepar--ations of B. subtilis 3610 (Glaser and Burger, 1964). Maximal incorporation of glucose residues into the glucose and N-acetyl glucosamine substituted teichoic acid of S. aureus occurred in conjunction with active synthesis of the polyribitol chain, indicating incorporation of glucosyl groups into the growing chain

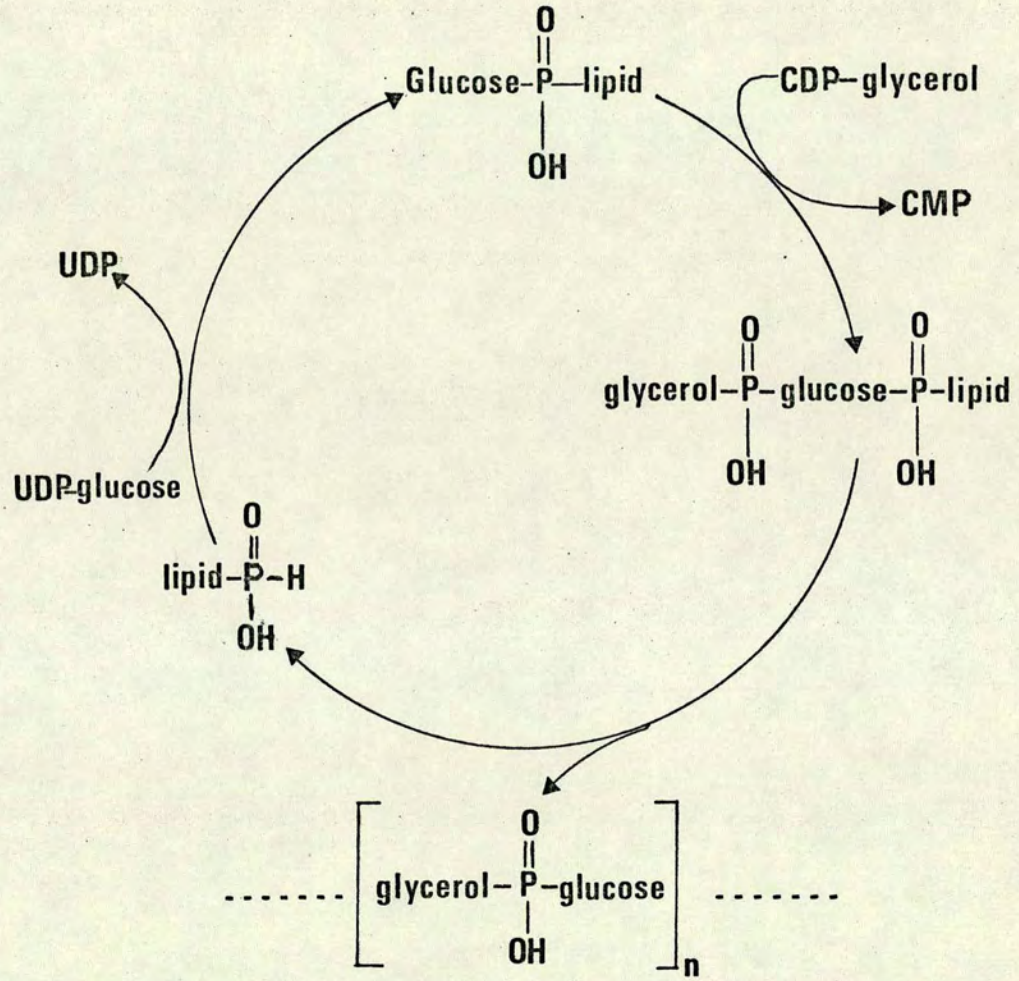


Fig. 10 Biosynthesis of the glucosyl-glycerol phosphate cell wall teichoic acid from Bacillus licheniformis ATCC 9945

(Ishimoto and Strominger, 1967).

The synthesis of teichoic acid from B. licheniformis (fig. 10) involves the formation of an unusual intermediate. The initial reaction comprises the transfer of glucose (rather than glucose-1--phosphate) from UDP-glucose to lipid. This is followed by the transfer of glycerol-1-phosphate from its CDP-derivative (Hancock and Baddiley, 1972). Thus the intermediate has no pyrophosphate linkage and bacitracin would not be expected to inhibit its synthesis.

(ii) Lipoteichoic acids.

Attempts to demonstrate biosynthesis of lipoteichoic acid have proved unsuccessful and therefore it is apparent that the biosynthetic mechanism differs fundamentally from that involved in cell wall teichoic acid synthesis. Since these polymers by definition contain lipid, the involvement of carrier lipids may not be required.

A group of membrane derived oligosaccharides comprising a polyglucose backbone with glycerol and phosphate substituents and therefore closely resembling membrane teichoic acids, have been studied (Van Golde, Schulman and Kennedy, 1973). Radioactivity was incorporated into water soluble polymer from chloroform/methanol extractible phosphatidyl glycerol. The product resembled authentic lipoteichoic acid and it is thought that this polymer may serve as acceptor in cell wall teichoic acid synthesis (see fig 11). However these compounds are found widely in gram-negative bacteria which contain no teichoic acid.

c) Biosynthesis of the linkage unit joining teichoic acid to peptidoglycan.

Attachment of de novo synthesised teichoic acid to peptidoglycan may occur either at the lipid intermediate stage or to the growing glycan chain, the two alternatives remain largely unresolved. However it is significant that glycerol phosphate is incorporated into polymeric material during conditions of active peptidoglycan synthesis. Additionally, synthesis of water soluble polymer containing glycerol, would not require active teichoic acid synthesis, providing endo--genous teichoic acid carrier remains in the preparation.

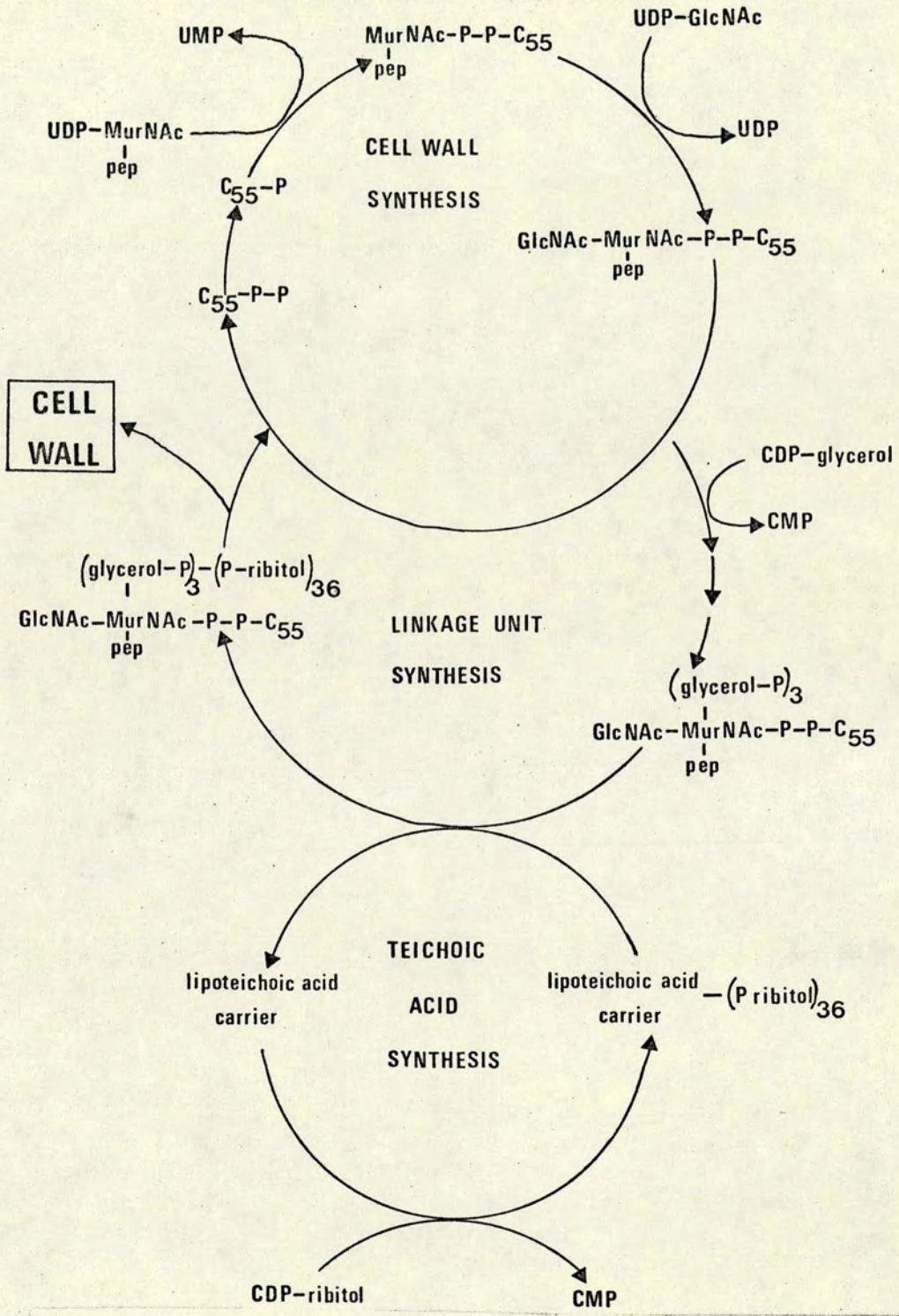


Fig. 11 Proposed mechanism for the biosynthesis of teichoic acid linkage unit and incorporation of teichoic acid into wall material : Hancock & Baddiley, (1976)

Hancock and Baddiley (1976) have demonstrated the incorporation of glycerol phosphate into a linkage unit in S. aureus H (fig. 11). Further studies have shown that the teichoic acids of several gram-positive bacteria are joined to muramic acid residues in peptidoglycan via a common linkage unit comprising one N-acetyl glucosamine phosphate and 3 glycerol phosphate residues (Roberts, McArthur, Hancock and Baddiley, 1979). The linkage unit is synthesised through the action of a series of polyprenyl pyrophosphoryl intermediates and from the precursors CDP-glycerol and UDP-N-acetyl glucosamine (fig 11) (Bracha and Glaser, 1976; Bracha, Davidson and Mirelman, 1978; McArthur, Roberts, Hancock and Baddiley, 1978).

SECTION 6 Lipopolysaccharide.

a) Structure.

The lipopolysaccharide found in gram-negative bacteria comprises by definition, polysaccharide and lipid moieties. Research into the chemical nature of lipopolysaccharide was initially stimulated by the discovery that these compounds possessed antigenic properties. Perhaps as a direct consequence, initial research favoured enteric organisms, particularly the genus Salmonella. With the finding that lipopolysaccharide may also serve as receptor site for bacteriophage, further weight was given to this research.

The general model of lipopolysaccharide structure comprises a lipid portion (lipid A) with endotoxic properties, a core region and the O-specific side chain, a region of repeating oligosaccharides often containing novel sugars. A generalised lipopolysaccharide from Salmonella sp is shown in fig. 12.

(i) Lipid A.

The lipid A region comprises a diglucosamine unit to which fatty acids are attached by ester and amide linkage. Phosphate and ethanolamine may also be present in variable proportions and in certain organisms, galactosamine may replace glucosamine (Reaveley et al., 1972). The major fatty acids vary from organism to organism and may provide taxonomic criteria. Fatty acids reported in Xanthomonas sp include 2-OH i-11:0 ; 3-OH i-11:0 ; 3-OH 12:0 ; 3-OH i-13:0 with a non-hydroxy fatty acid 10:0, i-11:0 (Rietschel, Lüderitz and Volk, 1975).

(ii) Core region.

The lipid A is linked by a labile ketosidic linkage to the reducing end group of ketodeoxyoctonate in the core region. Remarkably similar core structures have been reported from Salmonella strains with different O-serotypes. Major constituents include glucose, galactose, L-glycero-D-mannoheptose ketodeoxyoctonate and N-acetyl glucosamine with phosphate and O-phosphoryl-ethanolamine residues also being reported. With respect to core structure, Xanthomonas sp. differ from the salmonellae in that they are reported to possess uronic acid and glucose instead of heptose (Volk, 1968a). Certain pseudomonads also differ from the enteric generalisation;

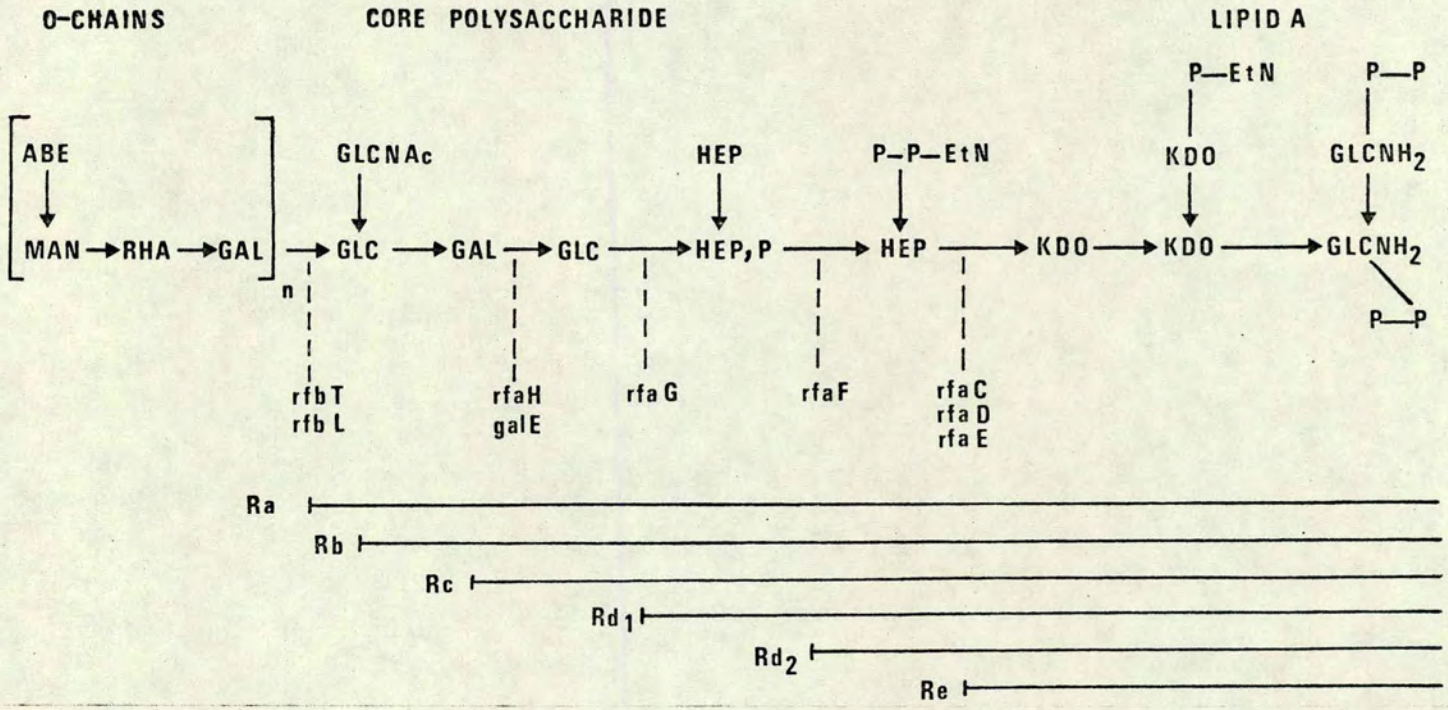


Fig. 12 Structure of the lipopolysaccharide from S. typhimurium: from Lüderitz, Galanos, Lehmann, Nurminen, Rietschel, Rosenfelder, Simon & Westphal (1973). Sugars still present in the LPS of chemotypes Ra-Re are shown by the solid line. Genes responsible for many of the synthesis steps are shown.

Ps. aeruginosa and Ps. alcaligenes contain alanine as an additional component.

(iii) O-antigen.

The specificity of the O-antigen is determined not only by the nature of sugar components but also the manner in which the sugars are linked. A wide variety of sugars has been detected, often forming an oligosaccharide chain of between 10 and 30 repeating units. Although the structures of many enteric O-antigens have now been elucidated, little is known of the structures of many others. The phenol solubilised core and O-antigen of Xanthomonas compestris has been reported to have an unusual composition with major components of rhamnose (58%) and 3-acetamido-3,6-dideoxy-D-galactose (25%). D-galacturonic acid (as the 1-phosphate) and glucose replace the core heptose and ketodeoxyoctonate (Volk, 1968a).

The water soluble lipopolysaccharide of several Xanthomonas sp. (Volk, 1968a; 1968b; Schlabach, 1970) has been reported to comprise the hexoses glucose and mannose, rhamnose, KDO and often either fucose or xylose (Volk, 1966; 1968b). Little is known of the structure, although the inner core region appears to contain a single KDO residue substituted with mannose phosphate (Volk, Salmonsky and Hunt, 1972).

b) Biosynthesis of O-antigen.

In most systems studied, biosynthesis of O-antigen is mediated through an isoprenoid lipid intermediate. The lipid involved in the biosynthetic mechanisms for O-antigen in Salmonella typhimurium (Weiner et al., 1965) and Salmonella newington (Wright et al., 1965) has been isolated and identified as undecaprenol phosphate. Much of the research on this subject has centred around these two organisms and their biosynthetic routes are essentially similar (fig. 13).

Initiation of synthesis occurs through the reversible transfer of galactose-1-phosphate from UDP-galactose to lipid with the release of UMP (Weiner et al., 1965; Wright et al., 1965; Dankert, Wright, Kelley and Robbins, 1966). Subsequent transfer of rhamnose and mannose from dTDP-rhamnose and GDP-mannose respectively, to the preformed galactosyl pyrophosphoryl lipid follows. Exogenous galactosyl-pyrophosphoryl-lipid and rhamnosyl-galactosyl-pyrophosphoryl-lipid serve as acceptors

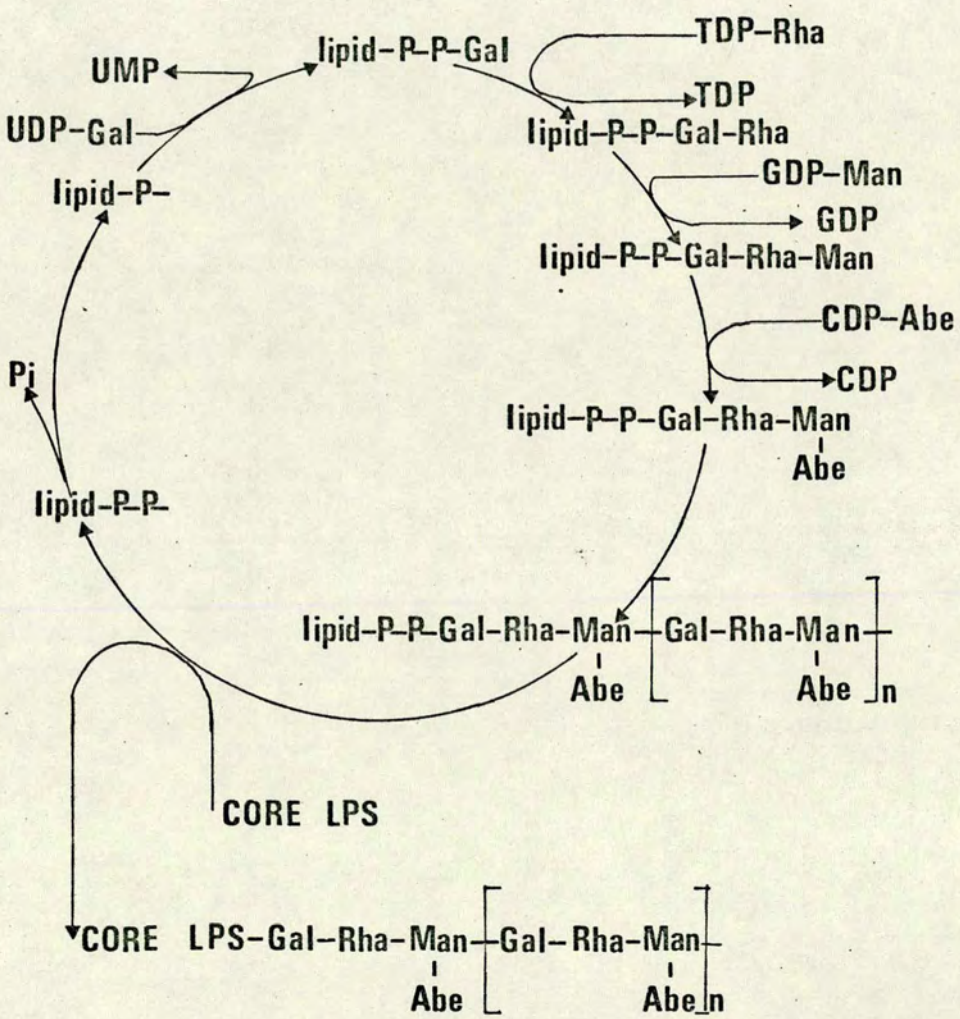


Fig 13. Lipopolysaccharide O-antigen biosynthesis in S. typhimurium

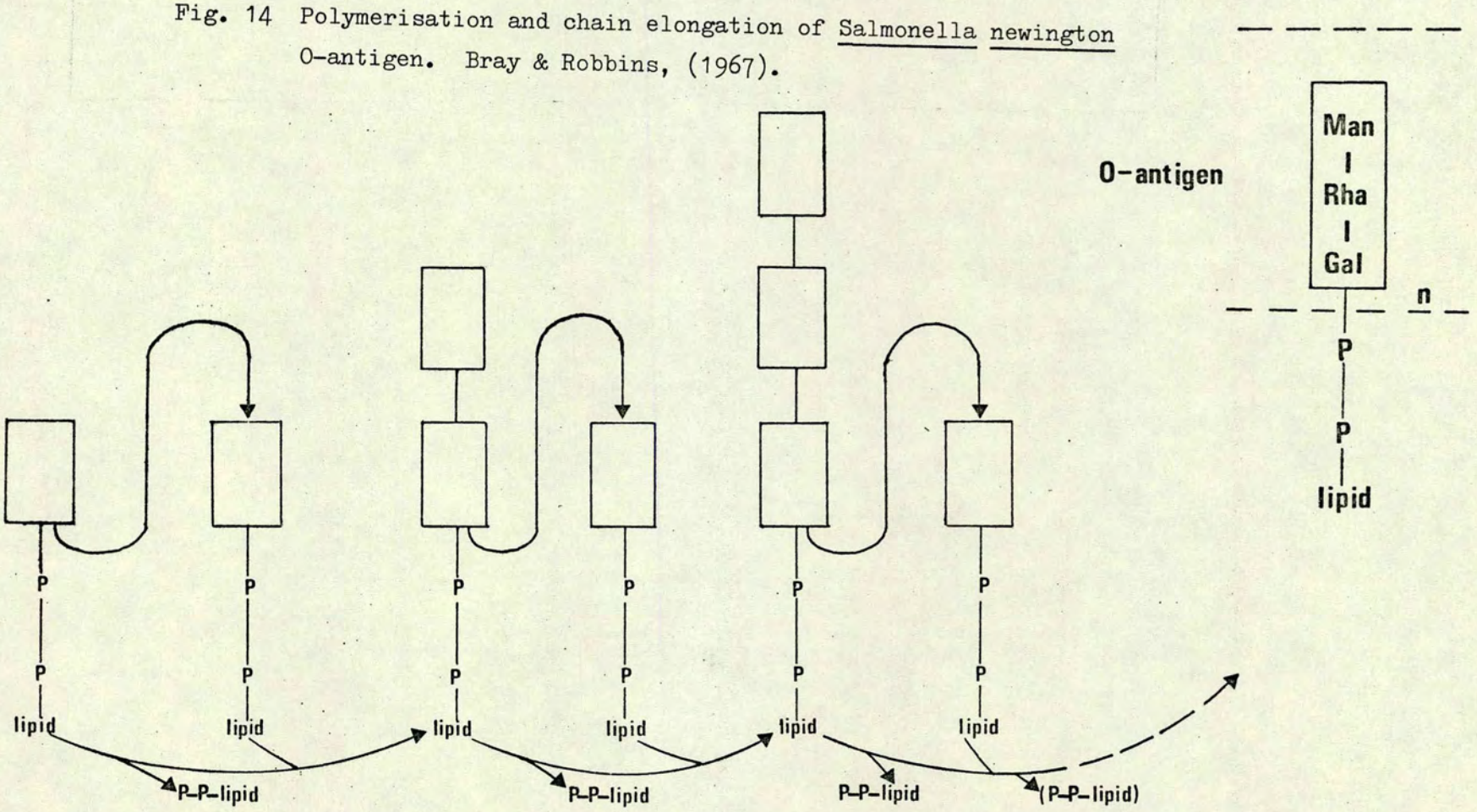
for rhamnose and mannose respectively (Kanegasaki and Wright, 1970). The additional monosaccharide side branch (see fig. 13) of abequose in S. typhimurium is donated to the trissaccharide-lipid from CDP-abequose (Osborn and Weiner, 1968).

Polymerisation of the tetrasaccharide repeating unit occurs when the unit is still attached to the carrier lipid and the octasaccharide thus produced has been isolated and identified (Osborn et al., 1968; Kent and Osborn, 1968). Pulse chase experiments have been used with great success in studying the polymerisation mechanism (Robbins, Bray, Dankert and Wright, 1967; Bray and Robbins, 1967). Growth of the chain occurs at the reducing terminus with polymer--isation taking the form of a series of stepwise transfers of repeating units (fig. 14). Fully polymerised chains are transferred to the acceptor, lipopolysaccharide core, with the release of lipid pyrophosphate. Using in vivo products, Nikaido (1969) established the linkage of O-chains as being to the distal glycosyl residue of the S. typhimurium core region.

As is usual with any generalisation, there are exceptions to this idealised biosynthetic mechanism. The 09 mannan lipopolysaccharide of E. coli is essentially a homopolysaccharide of mannose monomers and its synthesis proceeds without the involvement of isoprenoid lipid-linked intermediates (Kopmann and Jann, 1975; Flemming and Jann, 1978), but a butanol soluble glucolipid has been implicated (Kanegasaki and Jann, 1979).

Current knowledge of lipopolysaccharide O-antigen biosynthesis has without doubt owed much to the use of mutants blocked in specific steps in either biosynthesis or attachment of de novo synthesised polysaccharide. Inability to synthesise the tri- or tetrasaccharide unit may stem from the loss of a specific enzyme involved in sugar nucleotide precursor synthesis. Strains similarly defective in the production of nucleotide derivatives of galactose, rhamnose, mannose and abequose have been variously reported (Nikaido, 1961; Nikaido and Fukasawa, 1961; Nikaido, Naide and Makela, 1966; Rosen, Zeleznick, Fraenkel, Weiner, Osborn and Horecker, 1965). As yet, mutants defective in glycosyl transferase activity have not been reported.

Fig. 14 Polymerisation and chain elongation of Salmonella newington O-antigen. Bray & Robbins, (1967).



c) O-antigen modification.

The addition of glycosyl branches or O-acetyl groups to O-antigen is a common feature of bacterial lipopolysaccharide. Polymerisation is not affected by the presence or absence of such 'post-synthetic' modifications, whereas the addition of abequose side residues is a prerequisite for polymerisation.

The modification process in group B salmonellae is coded on the cell genome, however the analogous process in group E salmonellae is coded on lysogenic bacteriophage ε^{34}. This novel glycosylation reaction involves the transfer of glucose rather than glucose-1--phosphate from its UDP derivative to undecaprenol monophosphate. Thus a phosphodiester linkage is formed. The glucose residue is subsequently transferred to the trisaccharide-lipid intermediate (Wright, 1971).

A temperature sensitive mutant of phage ε^{15} produces a similarly temperature sensitive O-antigen polymerase. (Bray et al., 1967). At the higher, restrictive temperature, synthesis of lipid linked trisaccharide occurs as normal, however polymerisation does not occur. A comparable temperature sensitive mutant of Salmonella anatum has also been described (Losick and Robbins, 1967).

d) The site of O-antigen synthesis.

The activities of enzymes involved in O-antigen biosynthesis are associated with crude membrane preparations, as is carrier lipid. However available evidence shows that the majority of lipopoly--saccharide is located at the outer membrane (Shands, 1969; Parisi and Osborn, 1969; Rothfield et al., 1969).

Therefore synthesis of lipopolysaccharide at the cytoplasmic membrane would present the cell with difficulty, since either complete O-antigen or lipid-linked intermediates, must be transferred to the outer membrane by a specific translocase. Following transfer, no further monosaccharides can be added (Osborn et al., 1972). The discovery of periplasmic uridine-5′- diphosphate glucose hydrolase (5′-nucleotidase) in E. coli (Beacham, Kahana, Levy and Yagil, 1973) would suggest that in this organism at least, some sugar nucleotide substrate is available within the periplasm. This further suggests

that synthesis at the outer membrane may be a possibility, removing the necessity for a complex translocase system. Unfortunately no data concerning concentration and range of periplasmic sugar nucleotides has been presented.

SECTION 7 Exopolysaccharides.

Polysaccharides found outside the cell wall may be excreted as a continuous layer, closely associated with the cell surface or, as loose slime, excreted freely into the environment. The term exopolysaccharide is a general definition covering both forms of polymer. In the laboratory, the ability to produce copious amounts of exopolysaccharide is usually manifest in the production of large mucoid colonies on agar plates and by increased viscosity in broth cultures. The ability to produce exopolysaccharide is widespread.

In the laboratory extracellular polysaccharides need not be present in growing cells under all conditions and their presence does not seem to be necessary for survival of the cell. Exopoly--saccharides can be removed with relative ease or the ability to synthesise expolymer lost by mutation, with no adverse effect upon survival.

a) Structure.

Exopolysaccharides are produced from a limited range of monomers including certain hexoses, methyl pentoses, N-acetylated amino sugars and uronic acids. Pentoses are found only infrequently. In addition, several non-sugar residues have been reported eg ester linked O-acetyl, formate and succinate and ketal-linked pyruvate.

On the basis of monosaccharide composition, exopolysaccharides can be subdivided into two major groups, homopolysaccharides and heteropolysaccharides. Both groups may take the form of linear or branched polymers with,or without,non-sugar substituents and both types are found widely.

(i) Homopolysaccharides.

Several bacterial species produce homopolysaccharides often containing glucose as the single monosaccharide component. Strains of Acetobacter xylinum produce a linear polymer of $\beta 1 \rightarrow 4$ linked glucose residues, bacterial cellulose, comprising up to 600 mono--saccharides. Bacterial cellulose closely resembles plant cellulose, in structure but differs by its extracellular location in bacteria (Hestrin and Schramm, 1954).

Levans, polyfructoses of high molecular weight (one million or

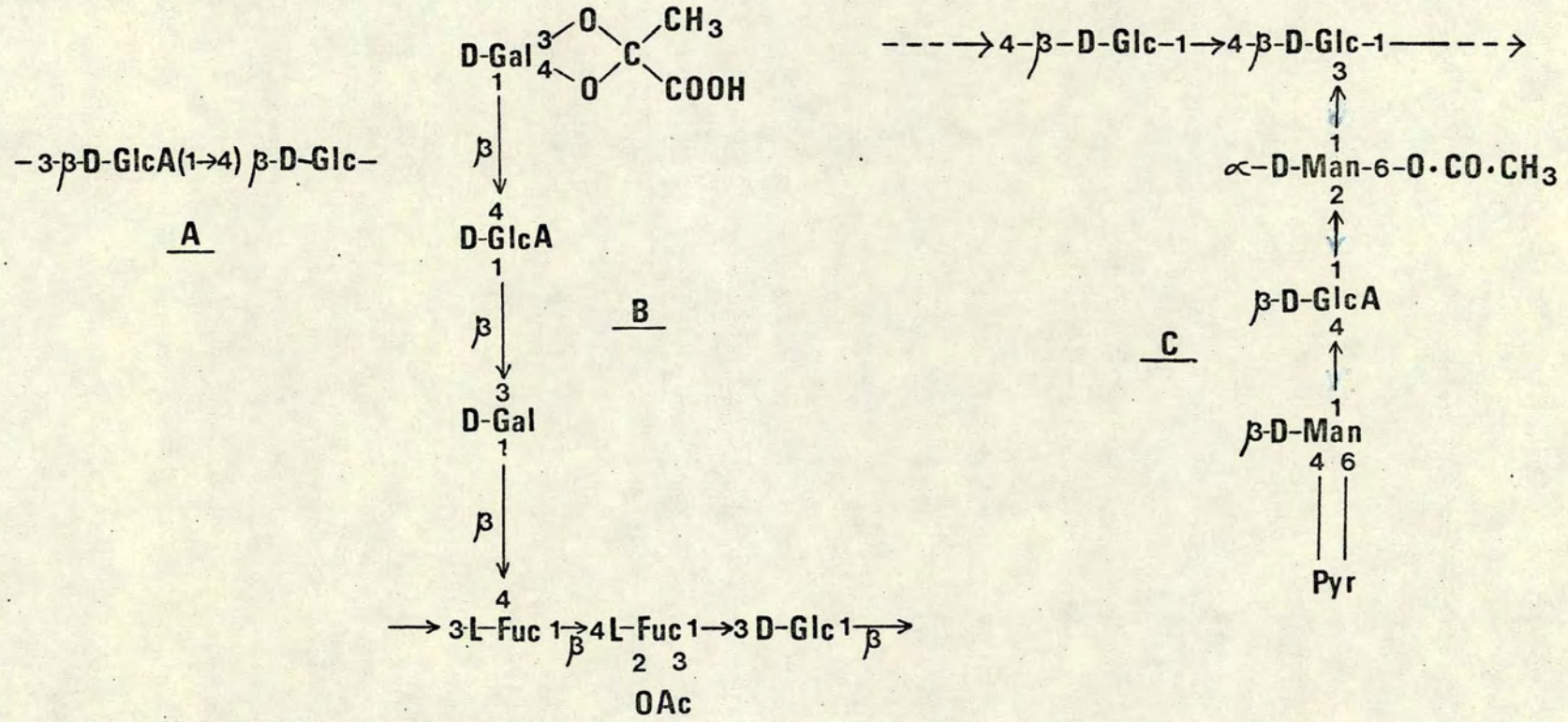


Fig. 15 Exopolysaccharide Structures: A S. pneumoniae type III (Adams, Reeves & Goebel, 1941) B Colanic acid from E. coli K12 (Sutherland, 1969; Garegg, Lindberg, Onn & Holme, 1971) C Xanthan gum from X. campestris (Jannson, Kenne & Lindberg, 1975)

greater) are branched polymers containing β-D-fructosyl 2→6 D--fructose as the predominant linkage (Cooper and Preston, 1935). Levans are produced by many plant pathogenic bacteria eg. Pseudomonas sp., strains of Aerobacter sp. and Acetobacter sp., members of the genus Bacillus and Streptococcus salivarius.

Dextrans are polymers comprising an α-D-glucosyl 1→6 D-glucose backbone with branching at positions 2, 3 or 4 (Peat, Schluchterer and Stacey, 1939). These polyglucose polymers are produced by a limited number of gram-positive bacteria including Leuconostoc mesenteroides L. dextranicum and Streptococcus viridans. Other polyglucose polymers have been identified in members of the genus Agrobacterium (Gorin, Spencer and Westlake, 1961) and contain β-D-glucosyl 1→2 D-glucose residues. Strains of Alcalignes faealis var. myxogenes produce polyglucoses in which residues may be linked in β1→3, β1→4 and β1→6 configurations (Harada, Masada and Fujimori, 1966). Under certain conditions however traces of galactose and succinate substit--uents have been identified (Misaki, Saito, Ito and Harada, 1969).

(ii) Heteropolysaccharides.

Heteropolysaccharides are composed of a regular repeating unit and with the ability to comprise more than a single monosaccharide constituent, a wide range of structures are possible. At simplest the repeating unit may be a disaccharide eg. Diplococcus pneumoniae type III (fig. 15A), but polysaccharides with varying degrees of complexity up to the level of a substituted heptasaccharide eg. E. aerogenes type 41 (Joseleau, Lapeyre, Vignon and Dutton, 1978) have been isolated.

A wide range of structures may be found in different strains of a single bacterial species. Polymers isolated from strains of E. aerogenes vary not only in monomeric components but also in the complexity of main and side chains and the degree of substitution by non-sugar residues.

The M antigen produced by E. coli and many other members of the Enterobacteriaceae (Grant, Sutherland and Wilkinson, 1969) has been named colanic acid (Goebel, 1963) (fig. 15B). Although the mono--saccharide composition of the trisaccharide side and backbone chains are largely invariable, slight differences may occur in the acyl

groups (Garegg, Lindberg, Onn and Sutherland, 1971; Garegg, Lindberg, Onn and Holme, 1971). Similarly, the exopolysaccharide produced by X. campestris may contain one acetyl and one ketal-linked pyruvate group per repeating unit (fig. 15C) but under certain conditions, the degree of pyruvylation is reduced (see below).

Bacterial alginates produced by strains of Azotobacter vinelandii (Gorin and Spencer, 1966) and Pseudomonas aeruginosa (Linker and Jones, 1964; 1966) are heteropolysaccharides formed from D-mannuronic acid and D-guluronic acid but lacking a regular repeating structure. The linear co-polymer is comprised of distinct blocks of polymannuronic acid and polyguluronic acid and a mixed block of alternating mannuronic and guluronic acid residues.

b) Production of exopolysaccharides.

Physiological conditions exert effects upon exopolysaccharide production, although different species are influenced by their environment in different fashions; no generalisations are possible.

Production of exopolysaccharide by E. aerogenes type 54 was maximum during sulphate, nitrogen (Duguid and Wilkinson, 1953) and to a lesser extent, phosphate (Duguid and Wilkinson 1954) limitation and minimum during oxygen or carbon limitation. Similar observations have been made with Chromobacter violaceum (Corpe, 1964) and Pseudomonas NCIB 1264 (Williams and Wimpenny, 1977; 1978). However in continuous cultures of A. vinelandii, limitation of sucrose, the carbon and energy source, had no effect on the rate of polymer synthesis (Jarman, Deavin, Slocombe and Righelato, 1978). Nitrogen-limited continuous cultures of X. campestris (Silman and Rogovin, 1972) and Pseudomonas NCIB 1264 (Williams et al., 1978) are also unaffected by further environmental change at dilution rates of 0.05-0.20h^{-1} and 0.025-0.25h^{-1}. respectively.

Strictly anaerobic bacteria eg Clostridium sp. and rumen bacteria (Baine and Cherniak, 1971; Cheng and Costerton, 1975) are capable of producing exopolysaccharides. In aerobic bacteria, aeration is an important parameter in production. In Xanthomonas phaseoli (Lilley, Watson and Leach, 1958) polysaccharide production increases with aeration up to 150 mm Hg, with no synthesis under anaerobic conditions. Similarly, aerobic conditions favour synthesis of exopolymer by

E. aerogenes (Duguid et al., 1953). Conversely, yields of polymer from Rhizobium meliloti (Dudman, 1964) were higher under conditions of low aeration; such conditions probably resemble the natural environment for these bacteria more closely. Similarly, yields of bacterial cellulose are reduced under conditions of high aeration. It is thought that catabolic pathways in A. xylinum are stimulated by aeration, thus reducing synthesis of sugar nucleotide precursors.

Exopolysaccharide synthesis is optimal at a pH of approximately 7 in cultures of Alcaligenes faecalis var. myxogenes (Harada, 1965), X. campestris (Moraine and Rogovin, 1966; Silman and Rogovin, 1970) and Pseudomonas NCIB 1264 (Williams et al., 1978). Nitrogen-fixing pseudomonads growing on low alcohols and glycols produce exopoly--saccharide more efficiently at pH 8 (Tanaka, 1974).

Addition of ions to cultures has been shown to exert an effect on the synthesis of exopolysaccharide and stimulation of synthesis can probably be linked to the ion requirements of specific enzymes. This phenomenon is particularly well documented in the case of divalent cations. Production of polymer by C. violaceum is stimulated by Ca^{++}, especially in the absence of Fe^{++} (Corpe, 1964). In E. aerogenes type 54 (Wilkinson and Stark, 1956) and Streptococcus pneumoniae type 3 (Bernheimer, 1953) production of polymer was stimulated by K^{+} and Mg^{++} but not NO_3^{-} and $SO_4^{=}$. In a recent study (Holt, 1978), the rate of synthesis of polymer production by X. campestris was increased by Ca^{++} (2 mM) and K^{+} (10 mM) however under similar conditions Zn^{++} (0.5 mM), Ni^{++} (0.5 mM), $PO_4^{\equiv}$ (1 mM) and NH_4^{+} (100 mM) were inhibitory. Furthermore, this study also showed that results obtained were dependent upon the nature of the washing buffer and therefore such results should be regarded with caution.

Exopolysaccharide can be produced at different phases of growth. For example, polymer is produced during logarithmic growth in cultures of Zoogloea MP6 (Unz and Farrah, 1976) and Ps. fluorescens (Eagon, 1956). In contrast, production of polymer by Pseudomonas NCIB 1264 does not begin until late log phase (Williams et al., 1977). Production of alginate by A. vinelandii (Deavin, 1976) is growth associated. In batch cultures of Ps. aeruginosa (Piggott,

1978) alginate production is also growth associated if the inoculum is taken from late stationary phase cells, but not if a younger culture is used as inoculum.

Despite the effect of environmental conditions on exopoly--saccharide synthesis, the product is generally independent not only of growth conditions but also, in the case of most heteropolysacc--harides, of substrate. Monomeric components of polymers from E. aerogenes (Wilkinson et al., 1955)and X. campestris (Davidson, 1978) are constant under a variety of conditions. Of interest however, is the finding that in both batch (Sandford, Pittsley, Knutson, Watson Cadmus and Jeanes, 1976) and continuous (Davidson, 1978) cultures of X. campestris, the degree of pyruvylation does vary. This finding may have a bearing upon the physical characteristics of this industrially important polysaccharide. Available evidence tends to suggest that the degree of acylation is variable in most heteropolysaccharides.

It is also interesting to note that although the molecular weight of exopolysaccharides differs between organisms, for a given organism grown under defined conditions, a product of remarkably uniform molecular weight is excreted (table 3)

c) Precursors.

In common with other cell surface polysaccharides, sugar nucleotides provide the precursors for exopolysaccharide synthesis by most species, with the exception of dextrans and levans (see below). However the common presence of acyl and ketal groups provides a need for further precursors.

In vitro work has established that acetyl CoA serves as the precursor for O-acetyl groups in Salmonella lipopolysaccharide (Keller, 1966) and in E. aerogenes type 8 exopolysaccharide (Sutherland and Wilkinson, 1968). Further studies with E. aerogenes type 8 (Sutherland et al., 1970) have established the precursors of acyl and ketal substituents to be acetyl CoA and phosphoenolpyruvate, respectively. Acetyl phosphate could not replace acetyl CoA as acyl donor. Addition of phosphonomycin, a structural analogue of phosphoenolpyruvate, resulted in inhibition of transfer of galactose from UDP-galactose to lipid and to polymer by membrane preparations

TABLE 3 Molecular Weights of Exopolysaccharides (Sutherland and Ellwood, 1979).

ORGANISM	MOLECULAR WEIGHT	REFERENCE
A. xylinum	5.67×10^5	1
X. campestris	2×10^6	2
	15×10^6	3
Streptococcus pneumoniae type III	2.67×10^5	4
E. coli K87	2.8×10^5	5
K. pneumoniae type I	2.94×10^6	6
E. aerogenes type 4	2.1×10^5	7
5	1.29×10^6	6
6	2.5×10^6	6
7	1.2×10^8	7
8	1.13×10^6	6
9	1.2×10^6	7
11	2×10^6	6
21	4×10^5	7
27	9.4×10^5	7
32	1.2×10^6	7
54	1.2×10^6	7
56	1.7×10^5	6
57	2.27×10^6	6
64	1.7×10^6	7
Pullularia pullulans	1.7×10^5	8

(see fig. 16). Inhibition was reversed by addition of further phosphoenolpyruvate. Addition of both acetyl CoA and phosphoenol--pyruvate simultaneously, stimulates the transfer of galactose, but not glucose, to lipid. Possibly the initial glucose residue carries an O-acetyl group or the galactose group carries both O-acetyl and ketal substituents and further addition of galactose residues cannot occur until acylation is complete (Sutherland 1977b).

d) Biosynthesis.

(i) Homopolysaccharides.

Biosynthesis of dextran homopolysaccharides has been widely studied and provides a useful example of a system which differs from the common 'carrier-lipid cycle'. Synthesis does not involve sugar nucleotides but is reliant upon supply of sucrose or an analogous oligosaccharide as carbon and energy source.

The extracellular enzyme dextransucrase catalyses the following reaction:-

$$n \text{ sucrose} \longrightarrow (\text{dextran})n + n \text{ fructose}$$

the product, of molecular weight approaching 5×10^5, (Sidebotham, 1974) is highly branched. No enzyme specifically con--cerned with branching has been isolated and it seems likely that dextransucrase functions both in branching and in chain elongation. The product may be closely associated with cell-bound enzyme in natural environments, indeed the 'insoluble' complex has been implicated in streptococcal plaque formation in experimental studies on rodents.

In vitro synthesis of bacterial cellulose by A. xylinum is dependent upon UDP-glucose as glucosyl donor (Glaser, 1958). Ethanol-soluble intermediates have been isolated (Colvin, 1959; Khan and Colvin, 1961; Manley, Jonker, Cooper and Pound, 1971) and tentatively identified as lipid pyrophosphate linked glucose and cellobiose. These intermediates result from the transfer of glucose-1-phosphate from UDP-glucose to lipid phosphate (Garcio, Recondo, and Dankert, 1974). These compounds are thought to be intermediates in cellulose biosynthesis. The significance of lipid monophosphate galactose, whose synthesis is favoured by the presence of Triton X-100, remains

unclear. Its structure was however subsequently confirmed as isoprenyl monophosphate galactose (Romero, Garcio and Dankert, 1977). The precise nature of the lipid moiety of glucosyl intermediates was not established, but the properties of these compounds also suggest a polyprenol. The final oligosaccharide chain length attached to lipid was not established, no intermediate larger than the cellobiosyl derivative was isolated, possibly as a result of the extraction techniques employed.

In a parallel study (Cooper and Manley, 1975a), in vitro synthesis of cellulose from glucose-1-phosphate was achieved. Addition of hydrolytic products of cellulose, cellodextrins, stimulated synthesis of cellulose from UDP-glucose (Cooper and Manley, 1975b) suggesting a function as initiator or chain acceptor.

In vivo cellulose synthesis has been visualised by electron microscopy (Brown, Willison and Richardson, 1976). A cellulose ribbon of up to 46 microfibrils is excreted from the pole of the cell. Use of negative-staining, sectioning and freeze etching techniques have shown the presence of some 50 synthetic sites, arranged in a row along the longitudinal axis of the cell. Recently, Zaar (1979) visualised pores in the outer membrane of A. xylinum using freeze--etching techniques. The authors suggested that these pores could provide a'passive' channel through the outer membrane or form an 'assemblyase' participating in the synthesis of the microfibril from nascent glucans.

(ii) Heteropolysaccharides.

Early studies of the sequences involved in bacterial hetero--polysaccharide biosynthesis were restricted to S. pneumoniae type III (Smith, Mills, Bernheimer and Austrian, 1960; Smith, Mills and Bernheimer, 1961). Without doubt, this early work was aided by ready characterisation of the product by immunological properties and susceptibility to a specific polysaccharase. Synthesis of hyaluronic acid by group A streptococci (S. pyogenes) from UDP-glucuronic acid and UDP-N-acetyl glucosamine,appears to follow a similar mechanism (Cifonelli and Dorfman, 1957). The synthetase enzymes have been reported to be bound to protoplast membranes (Markovitz and Dorfman,

1962). No intermediate beyond the nucleotide precursor has been reported and requirements for acceptors and primers were not established.

More recently studies with membrane preparations of E. aerogenes (Troy et al., 1968; Sutherland et al., 1970; Troy et al., 1971), have implicated a lipid intermediate in exopolysaccharide biosynthesis (fig. 16). The lipid has been established as the polyprenol, C_{55}-undecaprenol (95%) with traces of the C_{60}-homologue (5%). Lipid-linked intermediates containing tetrasaccharide (one repeating unit) and octasaccharide (2 repeating units) have been isolated and characterised.

The transfer of glucose and galactose from UDP-derivatives to ficaprenol has been demonstrated using butanol-soluble extracts from E. aerogenes (Lomax, Poxton and Sutherland, 1973). Glucose and galactose were transferred in a ratio of 1:0.81, possibly the second galactose transferase was not present, or was inactivated in the butanol extract (Sutherland, 1977b).

A similar lipid intermediate is involved in the synthesis of a colanic acid type polymer from E. coli (Johnson and Wilson, 1977). The lipid, tentatively identified as a polyprenol on the basis of hydrolytic properties and response to inhibitors, carries a sugar chain identified as (fucose $_3$, glucose $_2$) glucose.

The precursor of bacterial alginate is GDP-mannuronic acid (Pindar and Bucke, 1974). Initially, a chain of polymannuronic acid is synthesised. Subsequent conversion of mannuronic acid to guluronic acid is catalysed by an epimerase enzyme; no GDP-gulose has been isolated. The epimerase enzyme is extracellular in A. vinelandii (Larsen and Haug, 1970; 1971). A possible method of controlling epimerase activity has been suggested on the basis of epimerase-resistance conferred by O-acetylation of mannuronic acid residues (Davidson, Sutherland and Lawson, 1977). Synthesis of alginate by A. vinelandii occurs without the involvement of isoprenoid lipids and a particulate preparation synthesising poly--mannuronic acid from GDP-mannose, has been recently reported (Scott, 1979).

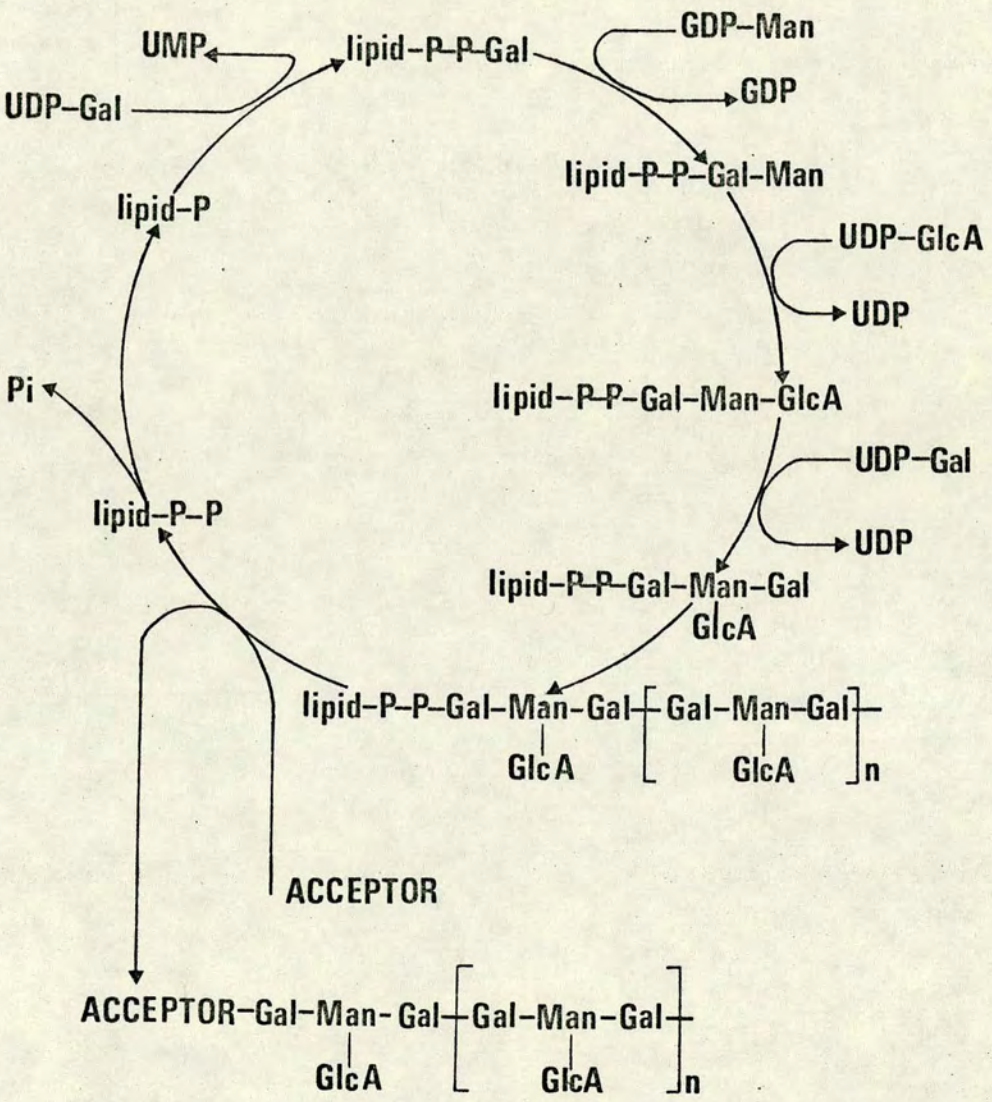


Fig. 16 Exopolysaccharide biosynthesis in E. aerogenes Troy, Frerman & Heath, (1971).

e) Control.

Control of exopolysaccharide synthesis can occur at a variety of levels. Most polysaccharides (with the exception of dextrans and levans) are synthesised intracellularly, thus the ultimate control of synthesis occurs at the level of substrate transport. Three types of substrate transport should be considered:-

(i) Facilitated diffusion, in which no transport can occur against a gradient.

(ii) Active transport, in which substrate transport is linked to respiratory chain-dependent oxidation of electron donors eg. D-lactate, hence a substrate can be pumped against a gradient.

(iii) Group translocation: sugars transported by phosphoenolpyruvate--dependent phosphotransferase systems are characteristically phosphorylated during the transport process.

In bacterial cells, a particular substrate may be transported by more than one system. For example S. aureus transports most, if not all, sugars by group translocation (Egan and Morse 1965a; 1965b), whereas E. coli and S. typhimurium may use all three processes. A single sugar may also be transported by more than one permease system eg. E. coli has four separate galactose permease systems (Rotman, Ganesan and Guzman, 1968). Studies on transport kinetics, coupled with mutant isolation have indicated that in most cases, the various systems function independently. However the isolation of pleiotropic mutants in apparently independent systems, tends to suggest a shared component (Postma, Cordaro and Roseman, 1977).

Of particular interest with respect to exopolysaccharide synthesis is the role of phosphoenolpyruvate-dependent group trans--location systems. If phosphoenolpyruvate is required for transport, what effect does this have on levels of intracellular phosphoenol-pyruvate available for pyruvylation of polymers?

Following transport, substrates may be lost to exopolysaccharide synthesis through catabolism, but may provide acyl substituents. Alternatively, sugar nucleotide precursors may be synthesised. In E. coli, the genes coding for sugar nucleotide synthesis are regulated by a plasmid, mutation to the mucoid phenotype may result from lesions in cap R, cap S or cap T genes. The three genes map at different loci

(Markovitz, 1977).

Control of the nucleotide sugar pool may be mediated through synthetic and hydrolytic enzymes (Ward and Glaser, 1968; 1969). Specific sugar nucleotide hydrolases have been identified in the periplasmic space in E. coli (Beacham et al., 1973) thus being spatially separated from at least some of the sugar nucleotides synthesised by the cell.

The fate of sugar nucleotides depends, at least in part, upon the phase of growth since the relative capability to synthesise the various surface polysaccharides, may differ with growth phase. Organisms capable of accumulating trehalose or glycogen, do so only in stationary phase and thus are unlikely to complete with any but exopolysaccharide synthesis. Separation of glycogen and exopolymer synthesis can then be facilitated by the use of ADP-glucose for glycogen and UDP-glucose for exopolymer synthesis. Further differ--entiation is possible through the involvement of lipid carriers in surface polysaccharide biosynthesis.

Synthesis of many surface polysaccharides requires the involve--ment of lipid intermediates. Evidence for the control of exopoly--saccharide synthesis at the lipid level is however, largely indirect and often inconclusive. A class of mutants from E. aerogenes has been described (Norval and Sutherland, 1969) in which production of normal lipopolysaccharide and exopolysaccharide, occurs at 37°C. At 25°C however, reduced amounts of lipopolysaccharide (30% of wild-type levels) are synthesised and exopolysaccharide is only produced in stationary phase. It has been suggested that at the lower restrictive temperature, isoprenoid lipid is limiting, with an order of priority being established. Priority would be given to essential peptidoglycan synthesis, followed by lipopolysaccharide and finally, exopoly--saccharide, which can be reduced or lost, with no apparent detrimental effect upon survival of the cell in the laboratory.

Further, indirect evidence for such priority has come from com--petition studies involving simultaneous peptidoglycan and teichoic acid synthesis in membrane preparations (Douglas et al., 1968; Watkinson et al., 1968; Anderson et al., 1971). Morphological

evidence may also support this suggestion; during cross wall formation in dividing bacteria, peptidoglycan synthesis always precedes lipopolysaccharide synthesis (Steed and Murray, 1966).

Possible "over-production of carrier lipid" has been suggested in bacitracin-resistant E. aerogenes mutants (Sutherland, 1977b). Higher levels of exopolysaccharide are produced in washed cell suspension and similarly, higher levels of transfer of glucose-1-phosphate from UDP-glucose to lipid phosphate were obtained in membrane preparations.

Finally, control may be exerted at the level of polymerisation and subsequent extrusion of the product from the cell. Synthesis of an exopolysaccharide with a substantial side chain eg polymers from X. campestris (fig. 15C) or E. coli (fig. 15B), may present difficulty to the cell. The definitive work of Troy and his colleages (Troy et al., 1971) has established that for a polymer with a single monosaccharide side chain, complete repeating units are assembled and partially polymerised, upon a lipid (fig. 16). However it is possible that longer side chains are constructed separately (cf lysogenic phage conversion of O-antigen; Section 6).

It is apparent that although early stages of exopolysaccharide synthesis leading to precursor and in some cases, intermediate synthesis are relatively well understood, little is known of the later stages of chain elongation and release. Recent work has indicated the possibility that discrete sites (about 20 per cell) localise the export of polysaccharide (Bayer and Thurow, 1977). Thus there are analogies with the excretion of lipopolysaccharide, which enters the wall at discrete sites rather than at random (Kulpa and Lieve, 1976). The action of 'ligases' is thought to release oligosaccharide chains from carriers, with simultaneous attachment to specific receptor sites. Loss of such receptors, for example by mutation affecting lipopolysaccharide, may result in the formation of slime, rather than capsule. How such a system could relate to an organism in which slime is the 'norm' remains unclear. An enzyme capable of reducing the degree of polymerisation of alginate has been isolated from certain alginate producing strains (Madgewick, Haug and Larsen, 1973).

This enzyme is thought to be involved in release of polysaccharide.

The mechanism by which the cell controls molecular weight (chain length) of the product also remains unclear. Whether this is a function of growth rate and determined by growth conditions, isoprenoid lipid availability, activity of ligase enzymes or due to specific polysaccharases, will only be resolved by further study. It seems likely that determination of chain length is governed by several factors working in unison, with the probability that such factors carry different importance in different organisms. For example polysaccharases have only been identified in alginate (see above), dextran and hyaluronic acid synthesising systems but most polysaccharides are not degraded by the organisms which produce them.

f) Function.

Despite the wealth of data now available on exopolysaccharide composition, structure and with certain reservations, biosynthesis, little is known of the precise function of these polymers in vivo. The finding that a high proportion of substrate available to the cell is diverted through biosynthetic sequences, leading to exopolysaccharide synthesis, together with the diversity of physiological conditions under which these polymers are produced, tends to suggest a major role in vivo.

Since bacteria are seldom capable of degrading their own exopolysaccharide to any extent, it is unlikely that they serve a storage function. A physiological role as an 'overflow metabolite' has been suggested from data obtained with E. aerogenes (Neijssel and Tempest, 1975; 1976). The biosynthesis of exopolymer in such a situation would be seen as a 'slip reaction' effecting turnover and hydrolysis of ATP, thus the exopolysaccharide is an'ATP-sink'.

Protection against phagocytosis and serum bactericidal factors, with loss of virulence occurring concomittantly with loss of cap--sulation, has been well documented (see Dudman, 1977). Protection of certain terrestrial and aquatic bacteria from the lytic activity of bacteriophage, slime-moulds, bdellovibrios and predatory protozoa has also been suggested. Indirect evidence suggests a further role in the protection against dessication in natural environments. The precise

mechanism of such protection remains unclear.

Adhesion of bacteria to surfaces is well documented in certain environments and this ability seems to be aided by the ability to produce acidic polysaccharides (Johnson, Bazzola, Schechmeister and Shklair, 1977). Furthermore in plant-bacterial relationships, (symbiotic and pathogenic) the chemical structure and physical conformation of exopolysaccharides may play an important secondary role in host recognition (Dazzo and Brill, 1977; Morris, Rees, Young, Walkinshaw and Darke, 1977).

MATERIALS AND METHODS

Bacterial Cultures and their Maintenance.

Xanthomonas campestris ATCC 13951 (designated T646 in this study) was obtained from the American Type Culture Collection. Both the wild type strain and mutants subsequently derived from it, were maintained at 4°C on nutrient agar slopes and subcultured at monthly intervals. Strains were stored as freeze-dried preparations.

Acetobacter xylinum 1375 was obtained from the National Collection of Industrial Bacteria, Aberdeen and was maintained at 4°C on Glucose-Peptone-Yeast Extract (GPYE) slopes.

Growth of Bacteria.

Liquid cultures were routinely grown in glucose broth ie nutrient broth (Oxoid, Ltd, Southwark Bridge Rd, London) containing 0.05%(w/v) D-glucose. One-litre quantities were contained in 2-litre Erlenmeyer flasks.

Enzyme studies were carried out on cultures grown in and fully adapted to, minimal medium (Table 4), with the appropriate carbon source at 2% (w/v). Incubation was carried out on a reciprocal shaker, cultures of 50 ml or 1-litre being contained in 250 ml and 2-litre Erlenmeyer flasks, respectively.

Exopolysaccharides were produced from cultures grown on nitrogen deficient Yeast Extract medium (YE) (Table 5), solidified with 1.5% agar. Five hundred ml aliquots of medium were contained in sterile shallow enamel trays (35 x 25 cm).

Xanthan degrading bacteria were grown in 'H salts medium' (Table 6) supplemented with 0.05% casamino acids and 0.1% xanthan (Sigma Chemical Co Ltd, Kingston Upon Thames, Surrey).

A. xylinum 1375 was grown in static broth cultures of GPYE medium (Table 7); 50 ml and 1-litre quantities were contained in 250 ml and 2-litre erlenmeyer flasks, respectively.

Fine Chemicals and Enzymes.

All chemicals and biochemicals were of the purest grades available and purchased from BDH Chemicals Ltd, Poole, England, Koch-Light Laboratories Ltd, Colnbrook, England or the Sigma Chemical Co Ltd, Kingston Upon Thames, England.

Commercially available enzymes were obtained from the Boehringer Corporation, (London) Ltd.

TABLE 4. Minimal Medium

Na_2HPO_4	3.0 g	
KH_2PO_4	3.0 g	
$(NH_4)_2SO_4$	0.3 g	
$MgSO_4.7H_2O$	0.2 g	
Carbon Source	20 g	autoclaved separately
Distilled water	1 litre	

trace elements:-	$FeCl_3.6H_2O$	16.7 mg/litre
	$CaCl_2.2H_2O$	0.66 mg/litre
	$ZnSO_4.7H_2O$	0.18 mg/litre
	$CuSO_4.5H_2O$	0.16 mg/litre
	$MnSO_4.4H_2O$	0.15 mg/litre
	$CoCl_2.6H_2O$	0.18 mg/litre
	H_3BO_3	0.10 mg/litre
	$Na_2MoO_4.2H_2O$	0.30 mg/litre

TABLE 5. YE Medium

Glucose	20.0 g	autoclaved separately
Casamino acids	1.0 g	
Yeast Extract	1.0 g	
Na_2HPO_4	10.0 g	
KH_2PO_4	3.0 g	
K_2SO_4	1.0 g	
NaCl	1.0 g	
$MgSO_4.7H_2O$	0.2 g	
$CaCl_2$	1 ml of a 1% solution	
$FeSO_4$	0.1 ml of a 1% solution	
Distilled Water	1 litre	

TABLE 6. H Salts Medium

$(NH_4)_2SO_4$	3.0 g	
Na_2HPO_4	3.0 g	
NaH_2PO_4	3.0 g	
$MgSO_4.7H_2O$	0.2 g	
$FeSO_4$	0.05g	
Trace elements	1 ml	
Xanthan polysaccharide	1.0 g	autoclaved separately
Distilled water	1 litre	

trace elements solution	$CaCl_2.2H_2O$	0.60 g
	$ZnSO_4.7H_2O$	0.18 g
	$CuSO_4.5H_2O$	0.16 g
	$MnSO_4.4H_2O$	0.15 g
	$CoCl_2.6H_2O$	0.18 g
	H_3BO_3	0.10 g
	$Na_2MoO_4.2H_2O$	0.30 g
	Distilled Water	1 litre

TABLE 7. GPYE Medium

Glucose	20.0 g
Difco bacto-peptone	5.0 g
Difco yeast extract	5.0 g
Distilled water	1 litre

The following radiochemicals were purchased from the Radiochemical Centre, Amersham, England:- D-[U-^{14}C]-Glucose (>230mCi/m mol); D-[U-^{14}C]-Mannose (2-5mCi/m mol); UDP-D-[U-^{14}C]-Glucose, Ammonium Salt (200mCi/m mol) GDP-D-[U-^{14}C]-Mannose, Ammonium Salt (100-200mCi/m mol)

Mutagenesis.

Washed, logarithmic phase cells were resuspended in 10ml phosphate buffer pH 7.0 containing 30-60 µg/ml N-methyl-N'-nitro--N-nitrosoguanidine (MNNG) and incubated at room temperature for 30 min. Washed cells were resuspended in nutrient broth and incubated at 30°C for a 12h recovery period before plating out.

Ethyl methane sulphonate (EMS) mutagenesis was carried out according to the method of Loveless and Howarth (1959), with a recovery period in nutrient broth before plating.

Gamma-irradiation of washed logarithmic phase cells was carried out using a ^{60}Cobalt source with 1-3 mins exposure. Mutagenised cultures were diluted and plated directly.

Preparation and Purification of Exopolysaccharides.

Following growth for 72h on trays of YE medium, bacteria were harvested by scraping off the medium with a glass spreader and resuspended in physiological saline (0.85% NaCl w/v) containing 4 drops of formalin per 100 ml., using a blendor. Bacteria were removed by centrifugation at 25,000g for 30 mins.

Exopolysaccharide was precipitated from the supernatant by addition of 3 vols. of ice-cold acetone; precipitated polymer was isolated and resuspended in distilled water. Purification was achieved by 48h dialysis against running tap water after which the exopolysaccharide was freeze-dried.

Preparation of Lipopolysaccharides.

Lipopolysaccharides were extracted from freeze-dried bacteria using the aqueous-phenol technique of Westphal and Lüderitz (1954). A 10%(w/v) suspension of cells in distilled water, was heated to 65°C and mixed with an equal volume of 90%(w/v) aqueous phenol, at 65°C. The mixture was maintained at 65°C and stirred vigorously for 10 mins. The two phases were separated by centrifugation at 5,000g below 10°C, after cooling on ice. The aqueous, upper phase

was retained and dialysed for 24h against running water, in order to remove any residual phenol. The contents of the dialysis sac were then reduced in volume by rotary evaporation at40°C. The lipopolysaccharide was then isolated as a clear gel pellet, following centrifugation at 100,000g for 4h at 4°C. Further purification of crude lipopolysaccharide was achieved by treatment with ribonuclease and deoxyribonuclease (0.01 mg/ml) followed by further dialysis. The crude lipopolysaccharide was freeze-dried

Partial Hydrolysis of Lipopolysaccharide.

10-20 mg amounts of crude lipopolysaccharide were hydrolysed in 2 ml of 1% (v/v) aqueous acetic acid in sealed glass ampoules, for 90 mins on a boiling water bath. Lipid-A, coagulated by this process, was removed by centrifugation and the purified lipopoly--saccharide was dialysed before freeze drying.

Hydrolysis of Polysaccharides.

Polysaccharides were hydrolysed by heating at 100°C for 18h in 0.25M sulphuric acid, in sealed glass ampoules. The hydrolysate was neutralised with excess Amberlite IR 410 resin (HCO_3^- form) and concentrated by rotary evaporation.

Cell Breakage and Preparation of Membranes.

Bacteria were harvested by centrifugation at 12,000g for 15 mins using an MSE High Speed 18 centrifuge and resuspended in 10 ml of 10mM phosphate buffer pH7.0. The cell suspension was disrupted by dis--continuous ultrasonic irradiation using an MSE ultrasonic disintegrator (100 watt) at maximum amplitude. The lysate was main--tained below 4°C on an ice/ethanol mixture.

Unbroken cells and cell debris were removed by two centrifugation steps at 7,000g for 15 mins at 0-4°C. Membranes were sedimented by centrifugation at 100,000g for 1h at 0-4°C in an MSE "Superspeed" 65 centrifuge and resuspended in ice cold buffer.

Polyacrylamide Gel Electrophoresis of Membrane Proteins.

Crude membrane suspensions were prepared from sonic lysates as described above. In order to solubilise membrane proteins in sodium dodecyl sulphate (SDS), the crude suspension was added to an equal volume of double strength sample buffer at 100°C, in order to give a

final concentration of:- SDS, 0.1%; bromophenol blue tracking dye, 0.002%; 2-mercaptoethanol, 0.1%; and 10% glycerol in 0.01M Tris-HCl pH 6.8. Samples of 25 μl containing 50 μg of protein were applied to the gel.

Ten per cent acrylamide gels with 0.27% bisacrylamide were prepared in cassettes (13 x 20 x 0.1 cm) comprising chemically clean glass plates; gels were prepared at pH 8.8. A 5% stacking gel (spacer gel) at pH 6.8, was layered upon the lower gel. Electrophoresis was performed in equipment supplied by Raven Scientific Ltd, (Haverhill, Suffolk) at an initial constant current of 10mA, until the tracking dye entered the lower gel and subsequently, at 20mA, until the tracking dye had migrated 75% through the gel (ca. 5h). Electrophoresis buffer contained Trizma base (Sigma), 6.0g/litre; Glycine, 28.8g/litre and SDS, 1.0g/litre in distilled water.

Gels were stained by immersion in the following solution for 2-3h :- Coomassie Brilliant Blue R250, 1.25g; Methanol, 227ml; Glacial acetic acid, 46ml; Distilled water to 500ml. Destaining was achieved by immersion in an aqueous solution containing methanol, 5% (v/v) and glacial acetic acid, 7.5% (v/v) with frequent changes of destaining solution.

Extraction and Separation of Sugar Nucleotides.

Cells were harvested by centrifugation, washed and resuspended in a small volume of physiological saline, to form a paste. Nucleotides were extracted by a modification of the method of Morikawa, Imae and Nikaido, (1964). The cell plaste was added to 5 volumes of boiling ethanol and the mixture maintained at 70°C for 10 mins with stirring. After cooling, cell debris were removed by centrifugation at 100,000g for 15 mins and re-extracted with ethanol. Combined supernatants were concentrated by rotary evaporation at 10-20°C.

Protein was removed by precipitation with an equal volume of isoamyl alcohol/chloroform (1 : 1). After shaking for 15 mins, the emulsion was separated by centrifugation at 14,000g for 15 mins at 4°C. The upper, aqueous phase was removed, treated with chloroform/ isoamyl alcohol again and pooled extracts were centrifuged at

18,000g for 30 mins at 0°C in order to give a clear extract which was stored at -60°C.

Separation of the nucleotide extract was achieved by chroma--tography on a column of Whatman ET 11 Ecteola cellulose according to the method of Nilsson and Sjunnesson (1961). Cellulose powder was suspended in 0.5M NaOH, with stirring under vacuum for 30 mins and subsequently washed on a sintered glass pad with distilled water, until the washings were neutral. The slurry was resuspended in 1.0M triethyl ammonium acetate buffer at pH4.0 and degassed under vacuum for 30 mins. A glass column (50 x 1.5cm) was packed with the slurry under gravity and equilibrated using the same buffer for 24h, at a flow rate of 25ml/h. The column was then washed with distilled water for 14h, at the same flow rate.

Nucleotide extract (ca. 5ml containing material from 10g cells) was applied to the column and eluted in distilled water. The effluent was monitored continuously at 260 nm, using an LKB 4701A Uvicord I and recorder. Any uncharged UV-absorbing material was eluted in distilled water. When absorption returned to zero, a buffer gradient, comprising 600ml distilled water contained in a mixing vessel and 600ml 0.5M triethyl ammonium acetate pH6.0 in a reservoir vessel, was applied. Fractions of 10ml were collected until all UV-absorbing material had been removed from the column. Tubes containing UV-absorbing material were pooled and freeze-dried for subsequent analysis.

Preparation of [^{14}C]-labelled Nucleotide Pools.

Cells were harvested from 1-litre of glucose broth, washed and resuspended in 200ml of minimal medium salts and cells were starved of glucose by incubation at 30°C for 60 mins. Labelled glucose (20ml 10% (w/v) D-glucose + 20μCi D-[^{14}C]-glucose) was added and the suspension reincubated for 2h. Labelled cells were harvested by centrifugation, thoroughly washed and then extracted in ethanol as described above.

Extraction of Isoprenoid Lipids.

Isoprenols were extracted as non-saponifiable lipids, according to the method of Gough, Kirby, Richards and Hemming (1970). Wet cells

from 10-litres of glucose broth were suspended in 1% methanolic pyrogallol (1ml/g cells) and 60% aqueous KOH (0.5ml/g cells) and the mixture was boiled under reflux for 45 mins. Following cooling, the mixture was diluted with 3 vols. distilled water and extracted three times with diethyl ether/light petroleum (bp 40-60°C), (1 : 1 v/v) The pooled extract contained unsaponifiable lipid and was washed with water to free the extract from alkali, before evaporation to dryness under nitrogen.

Non saponifiable lipid was applied to a column of activated florisil (1 x 25cm) and eluted with 100ml diethyl ether/light petroleum (bp 40-60°C), (1 : 49 v/v); 100ml diethyl ether/light petroleum (1 : 9) and finally 100ml diethyl ether. Polyprenols were found in the 1 : 9 eluate.

Preparation of [^{14}C]-labelled Oligosaccharide Lipids.

Cells were harvested from 1-litre of glucose broth, washed and resuspended in 50ml of minimal medium salts, supplemented with 10μCi D-[^{14}C]-glucose and cold carrier glucose at a final concentration of 2mg/ml. After incubation at 30°C for 60 mins, cells were harvested. [^{14}C] sugar-lipids were extracted from washed cell paste with 20 vols. chloroform/methanol (2 : 1 v/v), with stirring for 3h at room temperature according to the method of Troy, et al., (1971). The organic phase was partitioned against 0.2 vol. 0.9% (w/v) saline, as described by Folch, Lees and Sloane-Stanley, (1957). The organic phase was isolated by filtration through Whatman 1PS phase-separating paper, washed twice with water and evaporated to dryness under nitrogen.

Scintillation Counting.

Radioactive samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer Model 3330 (Packard Instruments Ltd., Caversham, Berks.). Paper samples were counted in PPO scintillation cocktail, (0.4% 2,5 diphenylphenoxazole in toluene) and dried organic extracts or small volume aqueous samples, in Toluene-Triton scintillation cocktail, (Toluene, 1.16 litre; Triton X-100, 500ml; 2,5-diphenylphenoxazole, 9.1g; 1-4-di-2-(5 phenoxazolyl)-benzene, 0.22g).

Chromatography.

Paper chromatography was carried out on Whatman No.1 or 3MM paper by descending chromatography. Thin layer chromatography (tlc) was carried out on plates of silica gel G, or on Whatman SG 81 silica gel impregnated paper.

Solvent A — Butanol/Pyridine/Water, (6 : 4 : 3,v/v); (Whistler and Conrad, 1954).

Solvent B — Pyridine/Ethyl acetate/Acetic acid/Water, (5 : 5 : 1 : 3,v/v); (Fischer and Dörfel, 1955).

Solvent C — Ethyl acetate/Acetic acid/Formic acid/Water, (18 : 3 : 1 : 4, v/v); (Feather and Whistler, 1962).

Solvent D — 1.OM Ammonium acetate/Ethanol, (3 : 7, v/v; pH 7.5); (Paladini and Leloir, 1952).

Solvent E — Saturated ammonium sulphate/1.OM Sodium acetate, pH 7.5/Isopropanol, (80 : 12 : 2, v/v); (Randerath, 1962).

Solvent F — Chloroform/Methanol/Water, (65 : 25 : 4, v/v); (Weiner, et al., 1965).

Solvent G — Diisobutylketone/Acetic acid/Water, (60 : 45 : 6, v/v).

Solvent H — Petroleum ether (bp. 60-80°C)/Diethyl ether/Formic acid, (75 : 25 : 1 v/v) (V A Letts, personal communication).

Solvent J — Chloroform/Methanol/Acetic acid/Water, (80 : 25 : 5 : 3.6, v/v); (V A Letts, personal communication).

Paper electrophoresis was carried out on Whatman 3MM paper, for 4h at 80-100 mA using Locarte high voltage equipment irrigated with the following buffer:- Pyridine/Acetic acid/Water, (5 : 2 : 43, v/v; pH 5.3); (Sutherland, Luderitz and Westphal, 1965).

Detection of Material on Chromatograms.

Sugars were detected by immersion in alkaline silver nitrate reagent (Trevelyan, Proctor and Harrison, 1950).

Nucleotides were detected by fluorescence at 260nm.

Lipids were detected on tlc plates by immersion in iodine vapour (Marinetti, 1964). The Dittmer reagent (Dittmer and Lester, 1964) was used to visualise phospholipids. Glycolipids and phosphatides were detected using the Periodate-Schiff reagent (Baddiley, Buchanan, Handschumacker and Prescott, 1956).

Polysaccharide Analysis by Gas Liquid Chromatography (GLC).

The component sugars of polysaccharide samples were analysed as their respective peracetylated aldonitrile (PAAN) derivatives, following acid hydrolysis (0.25M H_2SO_4/100°C/18h), according to the method of Linton and Cripps, (1978). Dried hydrolysates were subjected to successive treatments with i) 20mg hydroxylamine hydrochloride in 0.4ml dry pyridine at 100°C for 40 mins and ii) 0.5ml of acetic anhydride at 100°C for 10 mins. The solvents were evaporated and the PAAN derivatives dissolved in 0.2ml chloroform. Prior to analysis, each PAAN derivative was partitioned against $CHCl_3/H_2O$ (2 : 1, v/v) and the $CHCl_3$ layer retained for analysis.

GLC analysis was carried out at 195°C using a column (175 x 0.3cm) of 3% neopentyl glycol succinate on Chromasorb W-AW, using nitrogen carrier gas at a flow rate of 60 ml/min. The peak areas were computed by a Hewlett Packard 3380 integrator. Components were identified by their respective retention times in comparison with authentic standards.

Analytical Techniques.

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall, (1951).

Total carbohydrate was determined by the phenol-sulphuric acid reaction (Dubois, Gillies, Hamilton, Rebers and Smith, 1956).

Glucose and galactose were determined by glucose oxidase (Boehringer Corporation, London) and galactose oxidase (Worthington Biochemicals), respectively.

Uronic acids were determined by the Carbazole method (Bitter and Muir, 1962).

Pyruvate was estimated using commercial lactic dehydrogenase (Boehringer Corporation, London).

Acetate was measured according to the method of Hestrin (1949).

Physiological Enzyme Assays.

All enzymes were assayed in freshly prepared extracts at 30°C under optimal conditions of pH, substrate and cofactor concentration. Measurements were carried out in a Gilford Model 240 Spectrophotometer. Reaction mixtures were contained in silica-glass cuvettes of path length 1cm and containing a final volume of 1ml.

Specific activities were expressed as μmol of substrate con--verted/min per mg of protein. Reproducibility of the assays was within $\pm$ 10%. E_{340} for NADPH was taken as 6.22×10^3 l/mol per cm (Horecker and Kornberg, 1948). E_{600} for dichlorophenol indole phenol was taken as being 19.5×10^3 l/mol per cm, at pH 6.6 and 9.1×10^3 l/mol per cm, at pH 5.5 (Ng and Dawes, 1973).

The following enzymes were assayed according to the methods of Ng et al., (1973):- ATP-D-hexose-6-phosphotransferase (hexokinase), EC 2.7.1.1 ; ATP-D-gluconate-6-phosphotransferase (gluconokinase), EC 2.7.1.12 ; Glucose-6-phosphate dehydrogenase, EC 1.1.1.49 ; 6-phosphogluconate dehydrogenase (decarboxylating 6-phospho-D-gluconate-NADP oxidoreductase), EC 1.1.1.44 ; Isocitrate dehydrogenase, EC 1.1.1.42 ; Glucose dehydrogenase, EC 1.1.1.47 ; Gluconate dehydrogenase, EC 1.1.99.3 ; 6-phosphogluconate dehydratase, EC 4.2.1.12 and 3-deoxy-2-oxo 6-phosphogluconate aldolase, EC 4.1.2.14 (Entner-Doudoroff Enzymes); Aconitate hydratase (aconitase), EC 4.2.1.3 ; 2-oxogluconate kinase, EC 2.7.1.13 and 2-oxogluconate-6--phosphate reductase (oxogluconate metabolising enzymes).

Phosphoglucose isomerase, EC 5.3.1.9 and phosphomanose isomerase were assayed by modifications of the methods of Slein (1955).

Phosphoglucomutase, EC 2.7.5.1 was assayed by a modification of the method of Najjar (1955).

UDP-glucose pyrophosphorylase, EC 2.7.7.9 was assayed by a modification of the method of Munch-Peterson and Kalckar (1955).

GDP-mannose pyrophosphorylase, EC 2.7.7.e was assayed by a modification of the method of Munch-Peterson (1962).

UDP-glucose dehydrogenase (UDP-glucose NAD oxidoreductase), EC 1.1.1.22 was assayed by a modification of the method of Strominger, Maxwell and Kalckar (1957).

RESULTS

SECTION 1. Mutagenesis and Strain Characteristics.

a) Mutagenesis.

The production of copious amounts of exopolysaccharide by Xanthomonas campestris is manifest in the production of highly viscous cultures and by large mucoid colonies on solid media. The appearance of Xanthomonas T646 when grown on YE medium is shown in Plate 1. Microscopic examination shows that no discernible capsule is present, exopolysaccharide being excreted freely into the medium as slime.

At the beginning of this study, an attempt was made to isolate mutants of X. campestris defective in some aspect of exopoly--saccharide biosynthesis. Such mutants have proved highly successful in facilitating elucidation of pathways culminating in the synthesis of Salmonella lipopolysaccharide. Attempts to obtain mutants defective in growth or exopolysaccharide synthesis on selected hexoses (particularly glucose and mannose) proved unsuccessful, despite repeated treatments with MNNG, EMS, UV-irradiation δ-irradiation and 2-aminopurine.

Mutants producing little or no exopolysaccharide were not found to arise spontaneously, although several workers have suggested genetic instability in X. campestris (particularly in continuous culture), resulting in an increased population of non-mucoid bacteria (Cadmus, Rogovin, Burton, Pittsley, Knutson and Jeanes, 1976). However a variety of non-mucoid Xanthomonas strains were produced by mutagenesis; two of these isolates, designated 646NM1 and 646NM2 (Plate 2) were subsequently studied in greater detail. These mutants were stable ; no spontaneous reversion to the wild-type condition was detected. Furthermore, no reversion to the mucoid phenotype was achieved by subsequent treatment with chemical mutagens.

The absence of a selective tool for mutants proved a considerable handicap in the collection of mutants. As a result, selection could be made only upon the basis of colonial appearance. Such mutants were, by definition, a result of gross alterations with concomittant gross alterations in colonial morphology.

Three further mutants were selected on the basis of colonial

Plate 1 96h YE culture of Xanthomonas campestris 'wild-type' T646.

Plate 2 96h YE culture of Xanthomonas campestris non-mucoid mutant 646NM2

Plate 3 96h YE culture of Xanthomonas campestris crenated mutant 646D.

Plate 4 96h YE culture of Xanthomonas campestris crenated mutant 646E.

Plate 5 96h YE culture of Xanthomonas campestris crenated mutant 646KR

appearance. Xanthomonas 646-D was a spontaneous mutant, Xanthomonas 646E was isolated following EMS treatment and Xanthomonas 646KR after γ-irradiation. These strains were collectively termed 'crenated' mutants. The colonies produced by all three strains were large with crenated edges and almost granular consistency; the colonies appeared to have 'burrowed' into the surface of the agar. Colonies of 646D and 646E were yellow in colour (Plates 3 and 4) whereas 646KR was of paler colour (Plate 5). Mutation resulting in loss of yellow pigmentation was a frequent observation.

The crenated mutants were stable. No reversion to wild-type phenotype occurred either spontaneously or following mutagen treatment.

b) Growth of Strains.

X. campestris T646 produced significant amounts of exopoly--saccharide in shake-flask cultures of nitrogen-limited YE medium, containing 2% D-glucose and less viscous cultures in glucose broth. Growth curves for these media are shown in figs. 17A and 17B. Production of exopolysaccharide was estimated by determining the total carbohydrate content of cell-free supernatant samples, following 48h dialysis against running water. As a result, only high molecular weight material was measured.

Polysaccharide formation begins during logarithmic phase and continues into stationary phase, for up to approximately 40h in YE medium. A pH decrease of pH 7.0 to pH 6.5 occurred during growth of T646.

Growth curves for the non-mucoid mutants 646NM1 and 646NM2 were similar toT646 with respect to growth and pH decrease ; no significant levels of exopolysaccharide were recorded.

Growth curves for the crenated strains 646-D, 646-E and 646-KR were similar for each strain but differed significantly from T646 and the non-mucoid mutants when grown at 30°C. The growth curves for the representative 646E, grown in YE and glucose broth are shown in figs. 18A and 18B. Growth of the crenated strains occurs at a higher rate than in the wild-type strain, particularly in the complex nutrient broth medium. A decrease of pH from pH 7.0 to approximately pH 5.8

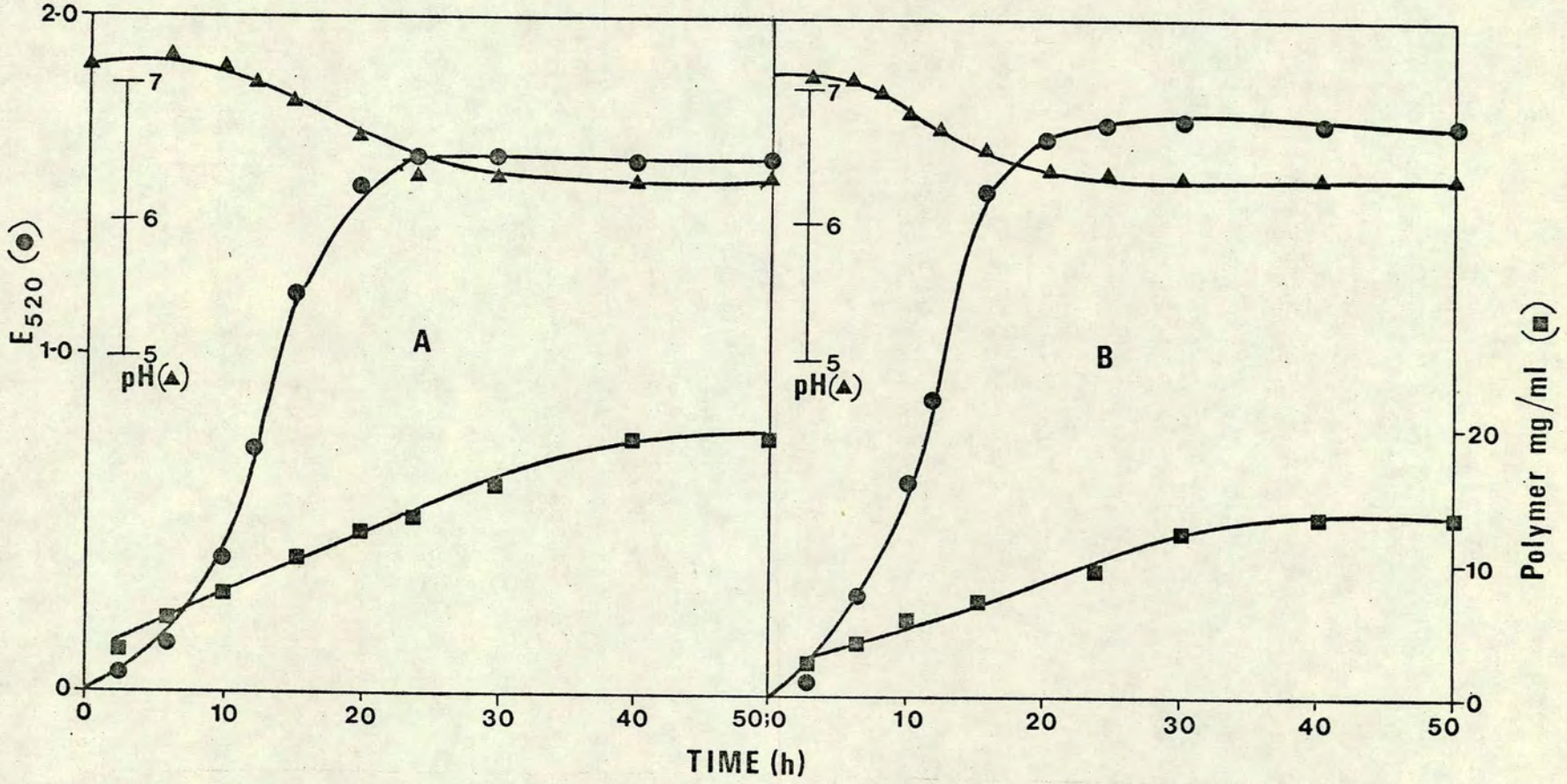


Fig. 17 X. campestris T646 grown in batch culture in YE medium (A) and Glucose Broth (B) at 30°C. (▲ pH, ■ polymer, ● E_{520}.)

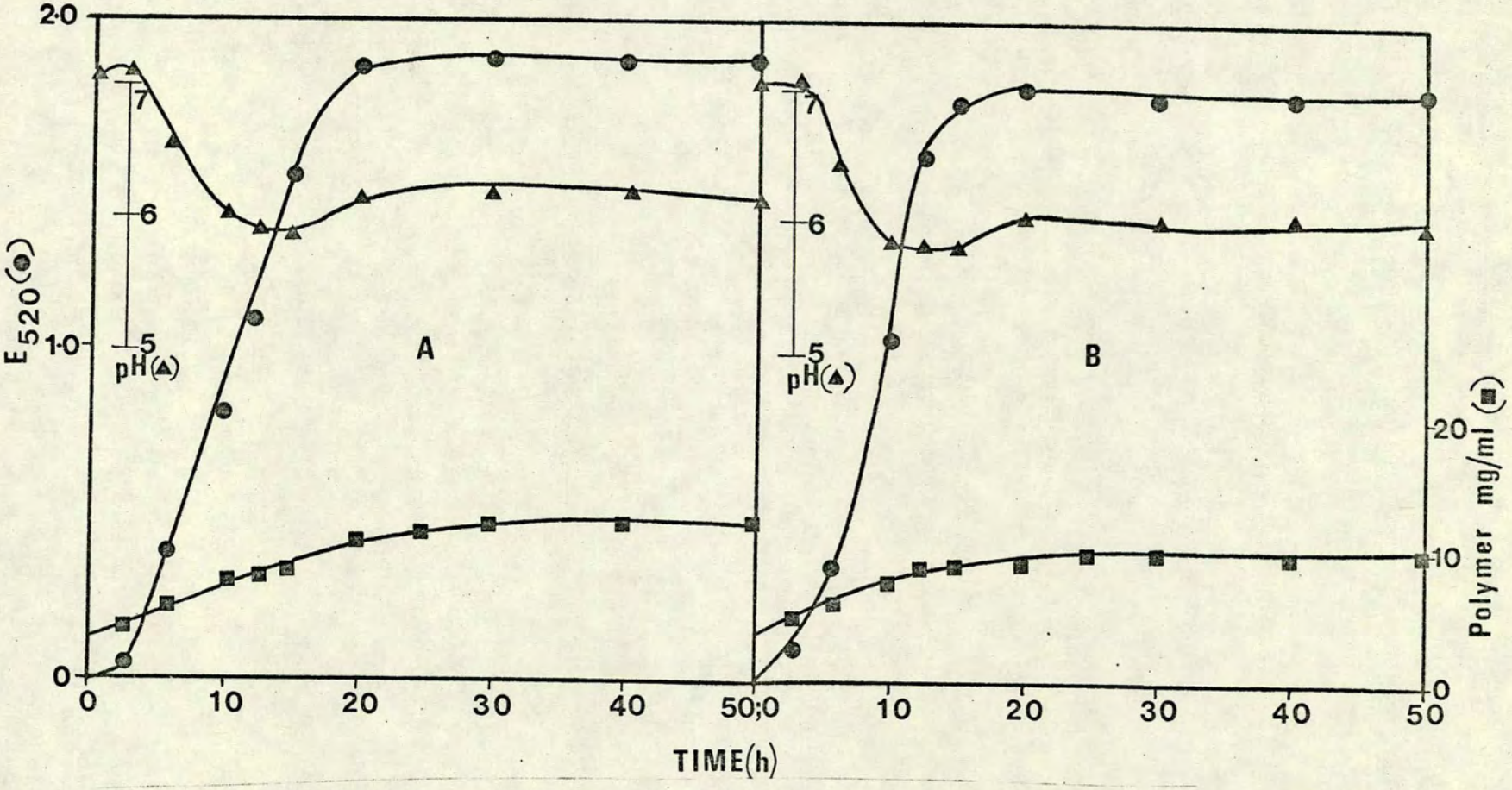


Fig. 18 Xanthomonas 646E grown in batch culture in the YE medium (A) and glucose broth (B) at 30°C (▲ pH, ■ polymer, ● E_{520})

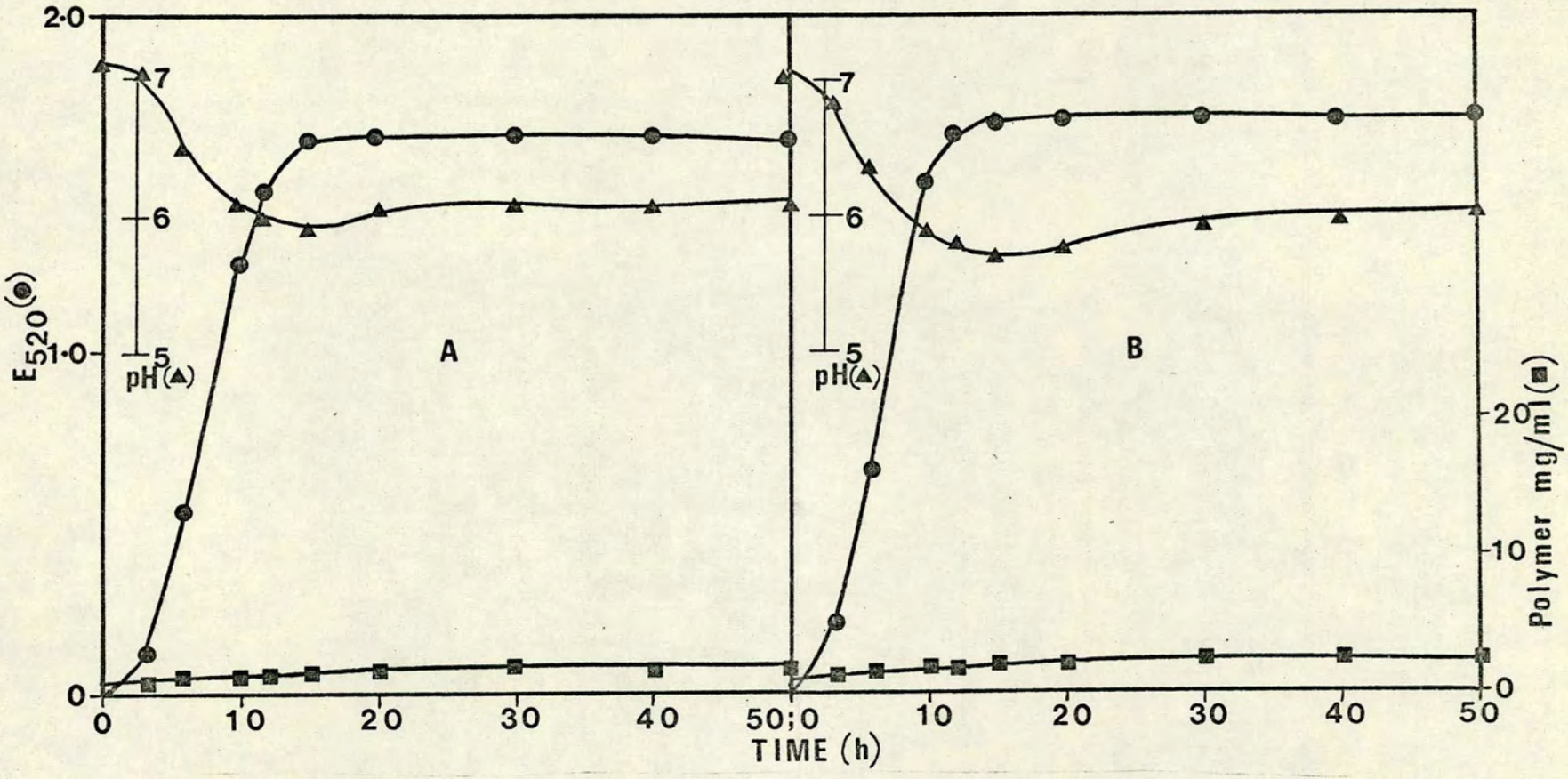


Fig. 19 Xanthomonas 646E grown in batch culture in the YE medium (A) and glucose broth (B) at 37°C (▲ pH, ■ polymer, ● E_{520})

occurs during growth of these strains. Synthesis of exopolysaccharide occurs at a lower level in the crenated mutants, when compared with the wild-type strain and as far as can be ascertained, synthesis of exopolysaccharide ceased after 30h in YE medium.

The crenated mutants also have the ability to grow at 37°C; T646, 646NM1 and 646NM2 are unable to grow at temperatures 32°C. Growth occurs at a similar rate in the crenated mutants at 30°C and 37°C and a corresponding decrease in pH is observed figs. 19A and 19B. Reduced levels of exopolysaccharide were synthesised at the higher temperature however. No significant difference was observed in the levels of polymer synthesis when crenated strains were grown in YE or glucose broth.

Strains of X. campestris were capable of growth and polymer production on a variety of substrates. Synthesis of exopolysaccharide was optimal when hexoses provided the sole carbon source, in all the strains studied. Levels of exopolysaccharide synthesis were lower during growth on disaccharides eg. maltose, cellobiose, lactose; sucrose however supported polymer synthesis at similar levels to hexoses eg. D-glucose, D-galactose, D-mannose.

Low levels of polymer synthesis occurred with the methyl-pentose, L-rhamnose as sole carbon source ; lower levels of synthesis also occurred during growth on organic acids eg succinate, pyruvate, citrate, gluconate and ketogluconate. In addition, low levels of polymer synthesis also occurred during growth on glycerol and glutamate.

c) Exopolysaccharide Synthesis During Growth.

Growth curves indicated that synthesis of exopolysaccharide occurred during growth of Xanthomonas strains. In order to determine the capacity to produce exopolysaccharide at different phases of the growth curve, washed cell suspensions of Xanthomonas T646 were used.

Samples were withdrawn from YE batch cultures and cells were harvested and washed twice in YE salts. Cells were finally resus--pended in YE salts containing 0.25 μCi/ml D-[^{14}C]-glucose with 0.25% cold carrier D-glucose; cells were suspended at a final concentration of 20 mg/ml (wet weight). Samples were withdrawn from the incubation mixture, cells deposited by centrifugation and aliquots of supernatant assayed for radioactivity following chromatography on

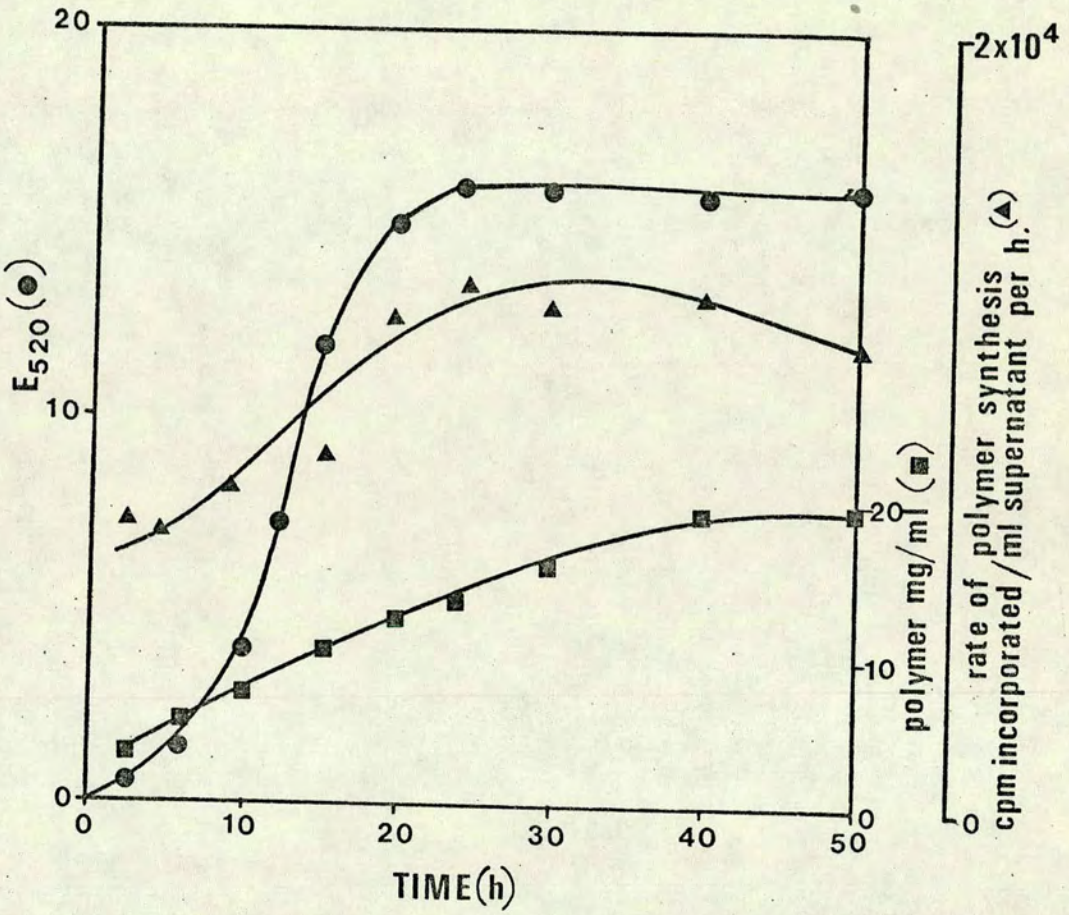


Fig. 20 Capacity to produce exopolysaccharide during growth of T646

● E_{520}; ■ polymer mg/ml; ▲ capacity to produce exopolysaccharide estimated in washed cell suspensions (see text).

Whatman 3MM paper in solvent D. After 24h irrigation, origins were dried and counted in PPO scintillant.

Results are illustrated in fig. 20. Optimal incorporation of radioactivity from D-[^{14}C]-glucose into polymer occurred during late logarithmic-early stationary phase. The rates of synthesis were lower in early logarithmic phase or during late stationary phase. No difference was observed in the timing of optimal polymer synthesis between T646 and crenated mutants studied in similar experiments.

d) Antibiotic Sensitivity of Xanthomonas Strains

The effect of several antibacterial antibiotics was tested against strains of X. campestris. The minimum inhibitory concentration for growth was estimated from a series of doubling dilutions. Dilutions were carried out in $\frac{1}{4}$oz. vials containing 1ml final volumes. Samples of overnight cultures were diluted 1 : 100 in sterile glucose broth containing diluted antibiotics. The lowest dilution at which growth was inhibited was taken as being the minimum inhibitory concentration for growth.

Several antibiotics were tested. Penicillin, bacitracin D-cycloserine and Vancomycin were chosen as antibiotics whose primary site of action is cell wall synthesis. The effect of Novobiocin is more complex. Although this antibiotic also affects cell wall synthesis leading to the accumulation of UDP-N-acetyl muramic acid--peptides, the incorporation of labelled intermediates into RNA, DNA and protein may also be inhibited. Novobiocin has been used to effect in the selection of mutants with altered cell surface com--ponents particularly lipopolysaccharides. The polypeptide antibiotic polymyxin B sulphate has a detergent action,which is active against the cell surface.

The sensitivities of Xanthomonas strains to each antibiotic is shown in Table 8. Crenated mutants were far more susceptible to growth inhibition by penicillin than were either T646 or 646NM2. In contrast, T646 was more sensitive to bacteria than were any of the mutant strains tested, a similar effect was seen with D-cycloserine, vancomycin and polymyxin B. The parent strain, T646 was capable of growth in higher concentrations of novobiocin than were any of the mutant strains.

TABLE 8. Antibiotic Sensitivities in Xanthomonas Strains : Minimum Growth Inhibitory Concentrations.

ANTIBIOTIC	T646	646NM2	646D	646E	646KR
Penicillin IU/ml	6,000	3,000–6,000	400	400	200
Bacitracin µg/ml	50–100	2,000	4,000	4,000	2,000–4,000
D-cycloserine µg/ml	100	200–400	200–400	200	200
Vancomycin µg/ml	50	200	200	100–200	200
Novobiocin µg/ml	100	50	50	50	25
Polymyxin B IU/ml	100	400	400	200	200

SECTION 2. Glucose Metabolism in Xanthomonas Strains

a) Enzymes Involved in Glucose Metabolism in Xanthomonas Strains.

Little literature is available concerning intermediary metabolism in Xanthomonas sp. Since pathways of carbon substrate catabolism may affect, directly or indirectly, the synthesis of exopolysaccharide, it was considered important that the situation be clarified.

Crenated mutants of Xanthomonas produced a marked decrease in the pH of the culture supernatant during growth (see Results Section 1). Consequently, growth of Xanthomonas 646D, 646E and 646KR on indicator plates containing bromothymol blue and glucose as sole carbon source, results in a colour change due to acid production. Under similar conditions, growth of Xanthomonas T646, 646NM1 and 646NM2 resulted in no colour change. Analysis of culture supernatants indicated the presence of significant levels of gluconic acid in the growth medium. These details, supported by the observ--ation that crenated strains and, to a lesser extent T646, 646NM1 and 646NM2, are capable of utilising gluconate and 2-oxogluconate as sole carbon sources, suggested the existence of an extracellular oxidative pathway, similar to that described for Ps. aeruginosa (fig. 21) (Ng et al., 1973)

The activities of the enzymes involved in glucose metabolism were tested in fresh extracts prepared from exponential phase cells, grown in minimal medium containing 2% defined carbon sources. Cells were grown in 1-litre quantities contained in 2-litre erlenmeyer flasks. Inoculum was taken from cultures fully adapted to growth on the appropriate carbon source. Incubation was carried out at 30°C on a reciprocal shaker.

Bacteria were harvested by centrifugation, washed twice with 67mM phosphate buffer, pH 7.0 and finally with 120mM glycyl-glycine buffer, pH 7.0 ; the washed cell pellet was finally resuspended in 60mM glycyl-glycine buffer, pH 7.0. Bacteria were lysed by sonication and debris removed by successive centrifugation steps at 7,000g for 15 mins ; the supernatant was used as a crude cell extract.

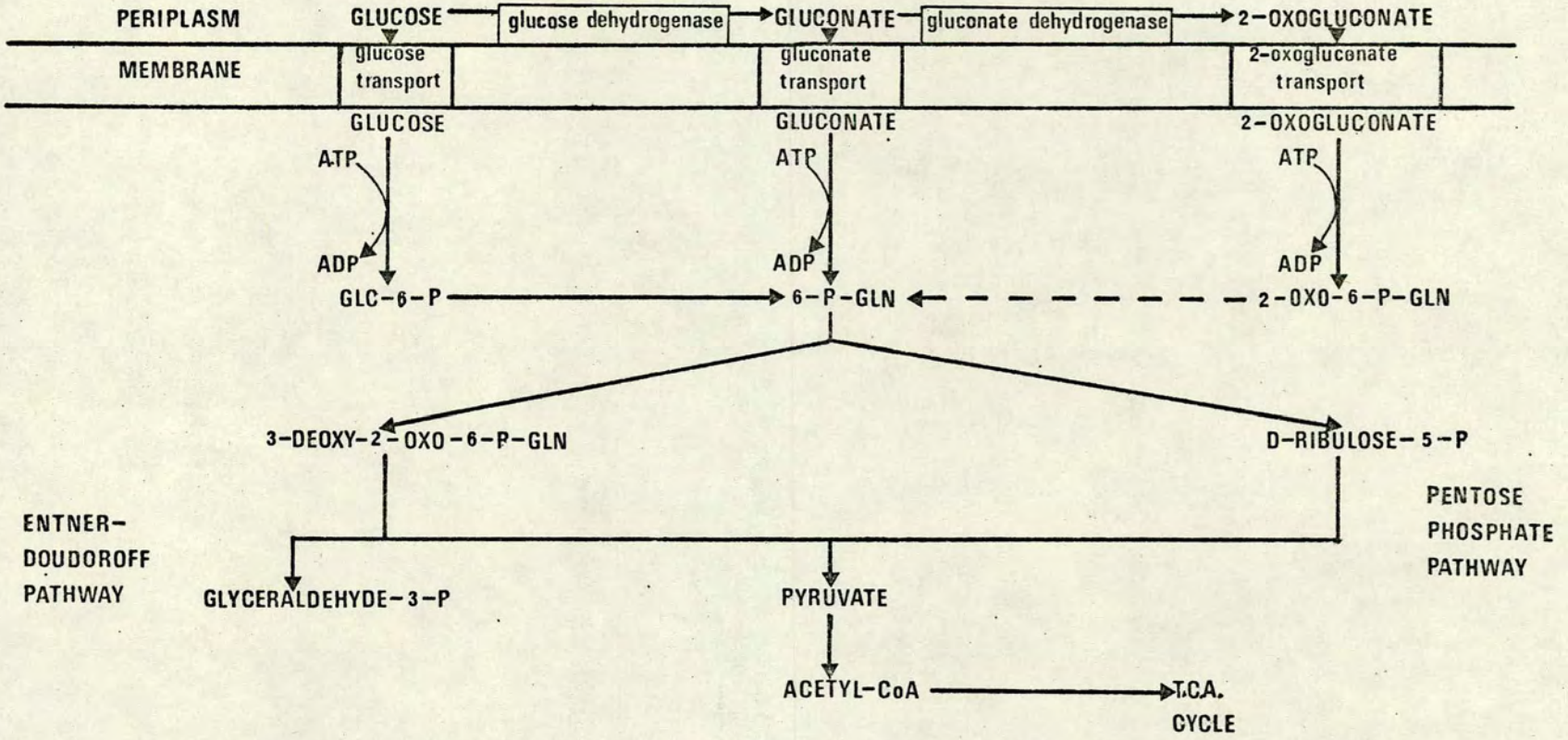


Fig. 21 Pathways of glucose metabolism in Pseudomonas aeruginosa, (Ng & Dawes, 1973)

Representative enzymes of the direct oxidative, phosphorylative, Entner-Doudoroff and Pentose Phosphate pathways were studied ; two enzymes (isocitrate dehydrogenase and aconitase) were taken as representatives of the Tricarboxylic Acid Cycle.

The activities of enzymes in extracts prepared from glucose grown Xanthomonas strains are indicated in Table 9. Differences are apparent between the wild-type organism and mutants derived from it. Neither Xanthomonas T646, nor non-mucoid mutants, possess detectable glucose dehydrogenase activity under the assay conditions employed whereas under the same conditions, glucose dehydrogenase activity was detected in 646D, 646E and 646KR. The amounts of gluconate dehydrogenase were appreciably higher in the crenated mutants, when compared with T646 and the non-mucoid mutants. Enzymes responsible for the metabolism of 2-oxogluconate were detected in all strains examined.

All these strains possess active enzymes of the phosphorylative (intracellular) pathway. Activities of the key enzymes of this pathway, namely hexokinase and glucose-6-phosphate dehydrogenase, vary widely.

The activities of Entner-Doudoroff enzymes were assayed in cell--free extracts by estimating the rate of pyruvate production ; pyruvate was measured enzymically using lactic dehydrogenase. Pyruvate was detected in all the strains examined although the amounts varied between strains. Pyruvate could not be produced by glycolysis under these conditions, since no activity of the key glycolytic enzyme phosphofructokinase was detected in any of the strains studied.

Crenated mutants of Xanthomonas exhibited detectable amounts of decarboxylating 6-phosphogluconate dehydrogenase, a key enzyme of the Pentose Phosphate pathway. No activity of this enzyme was detected in extracts prepared from either T646 or the non-mucoid mutants. The assay reaction relies upon the conversion of $NADP^+$ into its reduced form. Although absolute values for enzyme activity should be regarded with caution, the detection of activity for decarboxylating 6-phosphogluconate dehydrogenase in crenated mutants, when compared with the zero values obtained from extracts of T646,

TABLE 9. Specific Activities of Glucose Metabolising Enzymes of Xanthomonas campestris Strains. Cells were grown in 1-litre batch cultures containing minimal medium supplemented with D-glucose (2%); values given are the average of 2 determinations.

ENZYME	SPECIFIC ACTIVITY μmol/min per mg protein					
	T646	646NM1	646NM2	646D	646E	646KR
Glucose dehydrogenase	nd	nd	nd	10.7	7.8	4.6
Hexokinase	23.0	37.3	21.3	17.9	21.3	4.6
Glucose-6-phosphate dehydrogenase	144	253	252	129	116	107
Gluconate kinase	nd	nd	nd	nd	nd	nd
6-phosphogluconate dehydrogenase	nd	nd	nd	112	118	265
Gluconate dehydrogenase	6.1	5.1	7.3	11.4	10.9	11.6
2-oxogluconate metabolising enzymes*	14.3	16.9	38.3	40.2	56.1	41.3
Entner-Doudoroff enzymes **	11.4	7.8	6.3	14.5	12.1	7.7
Aconitase	119	88.5	52.6	46.2	43.7	40.2
Isocitrate dehydrogenase	245	231	290	231	221	210

nd, not detected.

* 2-oxogluconate kinase + 2-oxogluconate reductase

** 6-phosphogluconate dehydratase + 2-oxo-3-deoxygluconate aldolase

646NM1 and 646NM2, under the same assay conditions, is significant however.

The strains tested showed variable amounts of the two tricarboxylic acid cycle enzymes studied, aconitase and isocitrate dehydrogenase.

The same enzymes were also assayed in cell-free extracts prepared from T646 cells grown in minimal medium containing different carbon sources (Table 10). The defined carbon source was incorporated into the medium at a level of 2%.

No glucose dehydrogenase activity was detected in extracts prepared from cells grown on any of the substrates tested. Growth on gluconate resulted in a 4-fold increase in the amounts of gluconate dehydrogenase (when compared with values from glucose grown cell extracts); an appreciable amount of gluconokinase activity was measured. The activity of 2-oxogluconate metabolising enzymes was also increased in gluconate grown cell-extracts.

Growth on citrate resulted in a reduction in the amount of gluconate dehydrogenase ; no gluconate dehydrogenase activity was detected in glycerol grown cell extracts. The activities of 2-oxogluconate metabolising enzymes were reduced when cells were grown in media containing either glycerol or citrate.

The amounts of phosphorylative intracellular pathway enzymes, hexokinase and glucose-6-phosphate dehydrogenase were appreciably higher in extracts prepared from glucose and gluconate grown cells, when compared with activities of the same enzymes in glycerol or citrate grown cell extracts.

The activity of the Entner-Doudoroff pathway (ie pyruvate production) varied with substrate but the pathway was active in extracts prepared from cells grown on all the tested substrates. No activity of decarboxylating 6-phosphogluconate dehydrogenase or phosphofructokinase, was detected under any of the conditions studied. Growth on citrate and glycerol resulted in a reduction in the amounts of tricarboxylic acid cycle enzymes.

TABLE 10. Effect of Substrate on Glucose Metabolising Enzymes of *X. campestris* T646.
Cells were grown in 1 litre batch cultures containing minimal medium supplemented with 2% carbon sources; values given are the average of 2 determinations.

ENZYME	SPECIFIC ACTIVITY, μmol/min per mg protein			
	Glucose	Gluconate	Citrate	Glycerol
Glucose dehydrogenase	nd	nd	nd	nd
Hexokinase	23.0	26.3	20.3	16.1
Glucose-6-phosphate dehydrogenase	144	153	77.6	114
Gluconate kinase	nd	8.3	nd	nd
6-phosphogluconate dehydrogenase	nd	nd	nd	nd
Gluconate dehydrogenase	6.1	15.3	4.2	nd
2-oxogluconate metabolising enzymes *	14.3	19.1	6.4	4.9
Entner-Doudoroff enzymes **	11.4	6.6	5.5	11.5
Aconitase	119	140	55.2	83.1
Isocitrate dehydrogenase	245	128	115	77.3

nd, not detected.

* 2-oxogluconate kinase + 2-oxogluconate reductase

** 6-phosphogluconate dehydratase + 2-oxo-3-deoxygluconate aldolase

b) Glucose Uptake in Xanthomonas Strains.

Glucose uptake in Ps. aeruginosa is not mediated by phosphoenolpyruvate-dependent phosphotransferase systems (Phibbs and Eagon, 1970; Romano, Eberhard, Dingle and McDowell, 1970; Midgley and Dawes, 1973), unlike the Enterobacteriaceae (Kundig and Roseman, 1971a,b). Because of the possible implications on pyruvylation of exopolysaccharide (see Introduction Section 7), it was considered important to test for the activity of similar uptake systems in X. campestris.

Membrane vesicle systems have been used with great success in transport studies. The advantage of membrane vesicles lies in the fact that the majority (up to 99%) of cytoplasmic enzymes are released during preparation, thus effectively preventing subsequent metabolism of transported substrates. The vectorial properties and substrate specificities remain unchanged. (Konings, 1977). In addition, such systems can be supplemented with driving energy sources, or inhibitors . This can be achieved by sonication of vesicles in buffer containing the required compound.

Membrane vesicles were prepared from T646 cells by EDTA--lysozyme sphaeroplasting; modifications of the methods of Osborn, Gander, Parisi and Carson (1972) and Kaback (1971) were used. Attempts were made to stimulate possible phosphoenolpyruvate-dependent phosphotransferase systems capable of transporting D-[^{14}C]-glucose, D-[^{14}C]-mannose and D-[^{14}C]-galactose, by loading vesicles with concentrations of phosphoenolpyruvate up to 10mM or alternatively, to inhibit with the antibiotic fosfomycin (phosphonomycin), a phosphoenolpyruvate analogue. No phospoenol--pyruvate-stimulated, or fosfomycin-inhibited, transport of [^{14}C]--sugars was detected. This was determined by the absence of radio--active uptake into vesicles collected on a millipore filter, dried and counted for radioactivity in PPO scintillant.

This result may have been due to re-orientation of membranes during vesicle preparation, such that activity of phosphoenol--pyruvate-dependent phosphotransferase systems was not favoured.

However, available evidence (Konings, 1977) suggests that most vesicles are orientated in the same direction as the original cytoplasmic membrane, *in vivo*. Alternatively, excessive manipu--lation during preparation may have resulted in inactivation of binding proteins or transport systems. In order to test the preparative techniques used, vesicles were prepared from *E. coli* K12 using the same technique. The control vesicles accumulated radioactivity from D-[^{14}C]-glucose, furthermore, the observed uptake was stimulated by the presence of low, and increasing levels of phosphoenolpyruvate up to 1mM, under the same assay conditions used for T646 vesicles. Inhibition was however not achieved with fosfomycin at the levels studied (ie. 0-50μg/ml).

It therefore seems unlikely that the apparent absence of phosphoenolpyruvate-dependent phosphotransferase systems in *X. campestris*, was due to faults in preparative or assay techniques.

SECTION 3. The Cell Surface of Xanthomonas Strains.

a) Analysis of the Major Membrane Proteins.

Morphologically, differences were apparent between colonies of Xanthomonas T646 and the mutants studied. Furthermore, differences in antibiotic sensitivity possibly reflected changes in the cell surface; thus a brief examination of the membrane proteins of each strain was carried out.

Membranes prepared from Xanthomonas strains, grown and harvested under identical conditions, were solubilised in sample buffer at 100°C for two reasons; (i) in a previous report, outer membrane proteins of S. typhimurium were solubilised at 100°C but not at 37°C (Ames et al., 1974) (ii) SDS has been shown to activate protease activity (Pringle, 1975), use of a higher temperature would eliminate this problem. However the use of a high solubilisation temperature has been shown to reduce the electrophoretic mobility of at least one major outer membrane protein (Osborn et al., 1972; Inouye and Yee, 1973). Polyacrylamide gel electrophoresis was carried out as described in the methods section.

The results of gel electrophoresis of membrane proteins from Xanthomonas strains are illustrated in fig. 22. All the strains studied gave similar band patterns; however some finer differences were detected. Differences between strains (fig. 22 A-E) tended not to be in the absence of a particular protein (with a few, rare exceptions), but rather changes in the intensity of staining of a particular band, possibly indicating an increase (or decrease) in the concentration of a particular protein.

The pattern of proteins from strain 646-D membranes grown at 37°C (fig. 22 F) also indicated few, if any, gross changes but instead several marked changes in stain intensity. This profile was typical of the proteins from 37°C membranes of the crenated mutants.

Previous reports have indicated the presence of a large number of proteins in the cytoplasmic membrane, but fewer proteins in the outer membrane. A 1% solution of sodium lauryl sarcosinate was used to preferentially extract cytoplasmic membrane proteins from

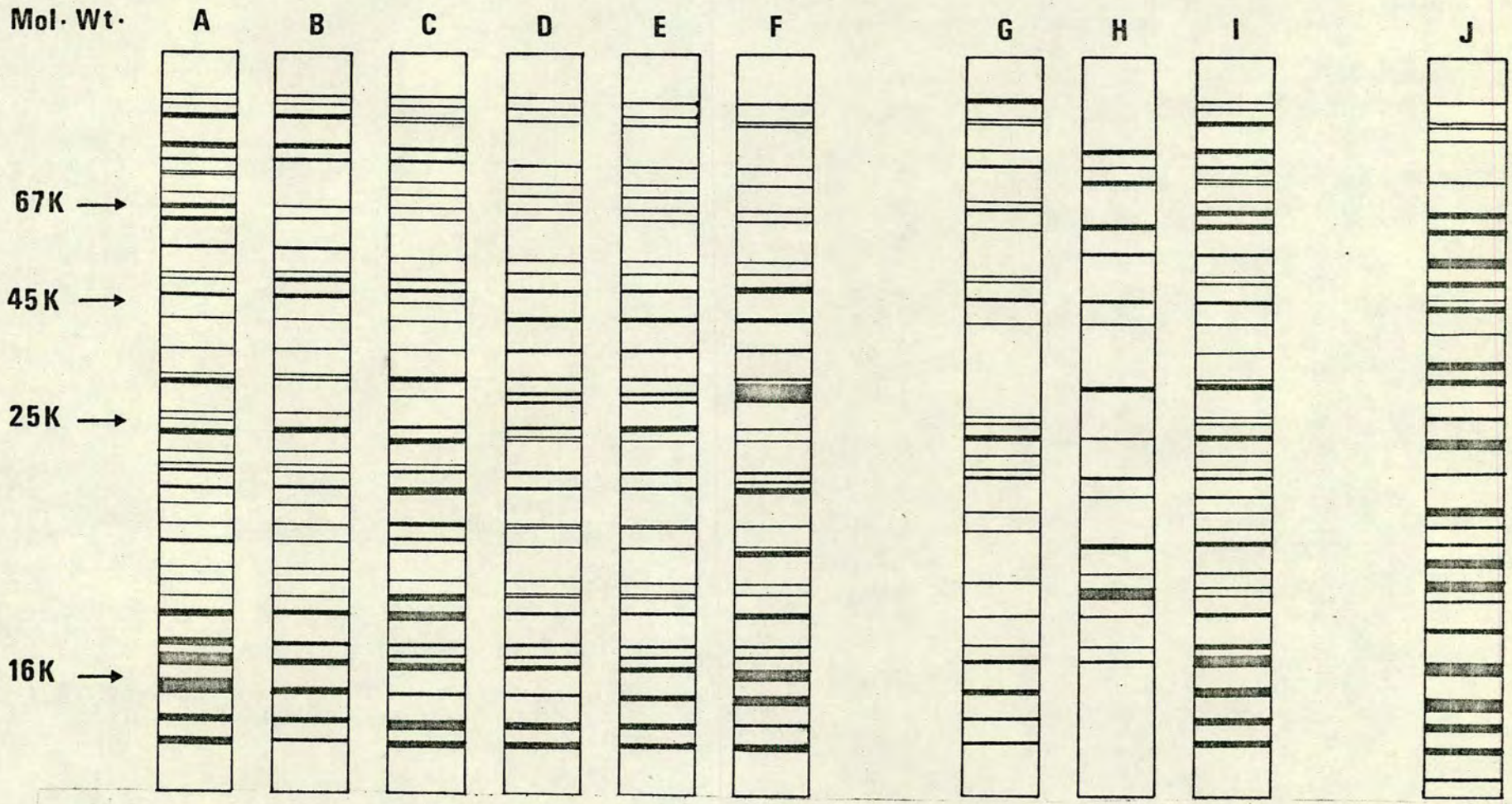


Fig. 22 SDS-polyacrylamide gel electrophoresis of membrane proteins A, T646 (30°C); B, 646NM2(30°C); C, 646E(30°C); D, 646KR(30°C); E, 646D(30°C); F, 646D(37°C); G, sarcosyl-soluble T646; H, sarcosyl insoluble T646; I, T646 Total; J, E. aerogenes strain A1X.

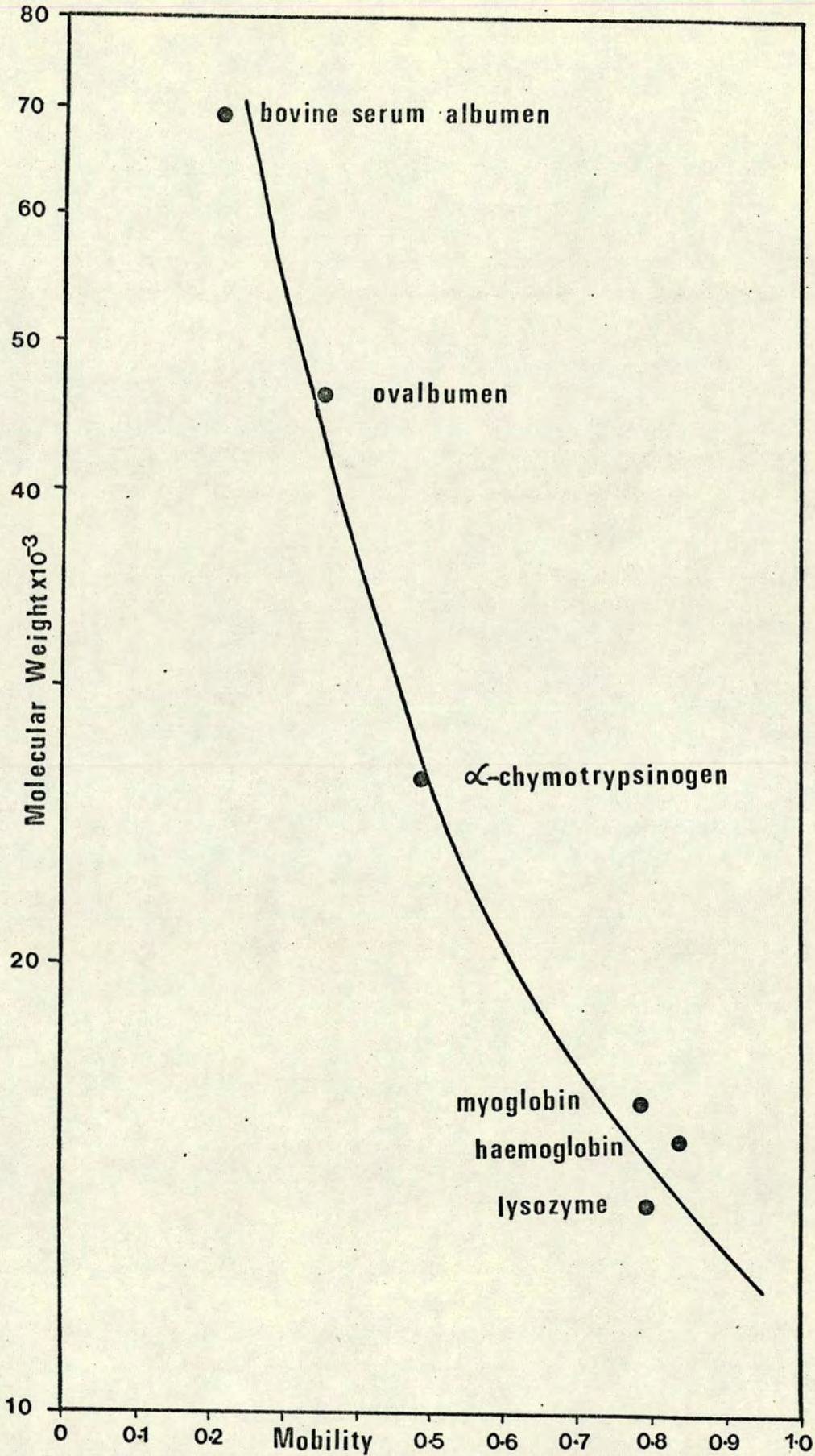


Fig. 23 Calibration curve for the determination of protein molecular weights using gel electrophoresis.

crude membrane preparations at 30°C for 30 min. (Filip, Fletcher, Wulff and Earhart, 1973). Insoluble material was deposited by centrifugation and solubilised in SDS. Sarcosinate was removed from the soluble preparation by dialysis against three changes of buffer at 4°C. Proteins became insoluble during this process and the material was resolubilised in SDS. The cytoplasmic membrane proteins (fig. 22 G) and outer membrane proteins (fig. 22 H) of Xanthomonas T646 are shown.

By way of comparison, fig. 22 J shows the band pattern obtained from a capsulate strain of E. aerogenes, separated under identical conditions (courtesy of G.D.Cumming). These results exemplify the differences between profiles from different bacterial species.

During this process, 30-40 proteins were routinely solubilised in SDS from Xanthomonas membranes. This figure is a conservative estimate since many of the intensely stained bands probably represent several polypeptides. Schnaitman (1970) has demonstrated that at neutral pH, several major outer membrane bands are resolved into several (as many as 4) distinct polypeptides.

Approximate molecular weights are shown for the membrane proteins. The molecular weights were obtained from authentic standards solubilised and subjected to electrophoresis under identical conditions. A calibration curve is illustrated in fig. 23.

b) Lipopolysaccharides.

In order to ascertain whether the described antibiotic sensi--tivity and membrane protein changes were associated with changes in the lipopolysaccharide layer, a detailed analysis of the aqueous--phenol soluble lipopolysaccharides was undertaken. Furthermore, it was considered important to establish the composition of both exopolysaccharide and lipopolysaccharide in order to cast light upon the possible fate of common precursors.

The yields of lipopolysaccharides extracted from freeze-dried cells using aqueous-phenol at 65°C (Table 11.), were higher in the crenated mutants than in the parent T646 or in 646NM2. The values were higher than expected and it is possible that the

TABLE 11. Lipopolysaccharide Yield Data.

STRAIN	GROWTH TEMP °C	YIELD OF CRUDE LPS FROM FREEZE-DRIED CELLS (% w/w)	YIELD OF POLYSACCHARIDE FROM LPS (% w/w)
T646	30	1.8	16.7
646NM2	30	3.9	51.4
646D	30	4.4	38.6
646D	37	4.9	27.6
646KR	30	8.3	39.4
646KR	37	7.6	40.9
646E	30	3.9	37.8
646E Soluble *	30	2.3	89.0

TABLE 12. Ratios of Sephadex G-50 Eluates.

STRAIN	TEMP °C	'O' ANTIGEN + CORE :	CORE POLYSACCHARIDE
T646	30	1 :	0.22
646NM2	30	1 :	0.26
646D	30	1 :	0.37
646D	37	1 :	0.61
646KR	30	1 :	0.36
646KR	37	1 :	0.40
646E	30	1 :	0.53
646E Soluble *	30	1 :	0.03

* see text

lipopolysaccharide preparation was heavily contaminated with nucleic acids (see below).

Polysaccharide fractions were purified by treatment with 1% acetic acid at 100°C for 1h. Coagulated lipid-A was removed by centrifugation. Yields of purified polysaccharide were higher in the crenated strains than in the parent T646, but lower than the values obtained for 646NM2.

Preparation of crude lipopolysaccharide involved sedimentation from dialysed extracts by centrifugation. In the crenated strains, a significant amount of polymeric material remained in the centri--fugation supernatant. This 'soluble' polysaccharide accounted for 2.3% (w/w) of the original extract in 646E. Following hydrolysis, a yield of 89% (w/w) purified polysaccharide was obtained implying that the soluble material was predominantly polysaccharide with little lipid-A.

Chromatography of polysaccharides on Sephadex G-50 (Super fine) facilitated separation of two major peaks of carbohydrate (detected according to Dubois et al., 1956). An initial peak of carbohydrate was excluded from the gel ; the high molecular weight material was presumptive complete polysaccharide ie O-antigen with attached core. The second, minor peak contained presumptive core material only.

In order to demonstrate the degree to which core material was substituted with O-antigen, samples of hydrolysed polysaccharide were chromatographed on a column of Sephadex G50 (40 x 2 cm). Pyridinium--acetate pH 5.4 was used as elution buffer ; fractions of 2ml were collected and aliquots analysed for total carbohydrate.

Elution profiles of lipopolysaccharides from T646 and 646NM2 are illustrated in fig. 24 A. The difference in the relative size of each peak tends to indicate a greater degree of O-antigen substitution in the parent strain. A similar analysis for 646E shows a further reduction in O-antigen substitution (fig. 24 B). Chromatography of the 'soluble' 646E polysaccharide revealed the presence of high molecular weight material, but little corresponding to core polysaccharide (fig. 24 B).

Peak material from each strain was pooled and freeze-dried

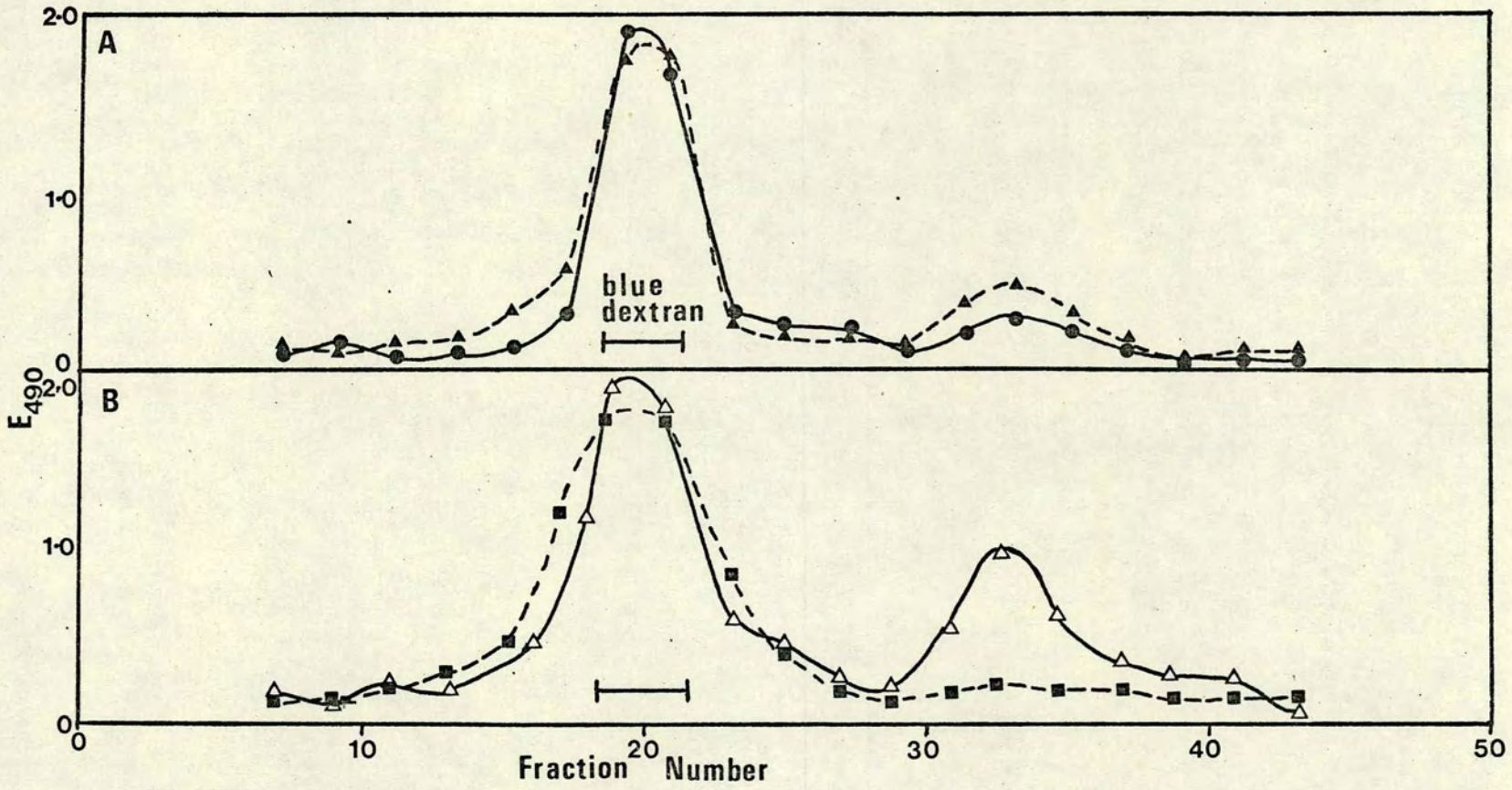


Fig. 24 Chromatography of partially hydrolysed lipopolysaccharides on Sephadex G50
A T646, ●–● ; 646NM2, ▲--▲. B 646E, △–△ ; 646E 'soluble' polysaccharide, ■--■.

before analysis for total carbohydrate. The results for each strain are summarised in Table 12. The figures obtained support the elution profile data and the suggestion that the crenated mutants contain less O-antigen than do T646 or 646NM2. Care must be taken in evaluation of these data since the nature and acid lability of the linkage between O-antigen and core has not been reported in X. campestris.

The component sugars in the purified polysaccharide of X. campestris were analysed following total acid hydrolysis (0.5N H_2SO_4 at 100°C for 16h). The presence of five major sugars was observed after paper chromatography (solvent A) : glucose, galactose, chamnose, mannose and ribose. No glucuronic acid was detected and only trace amounts of glucosamine were present.

Quantitative analysis was achieved by conversion of hydrolysed samples into PAAN derivatives, which were subjected to GLC as described in the methods section. It was assumed that the relative proportions of PAAN derivatives indicated the respective proportions of underivatised sugars in the original material and that detector response was equal for each PAAN derivative (Seymour, Chen and Bishop, 1979 indicated that the response for the hexoses was equivalent but that those for L-rhamnose and D-ribose were 93% and 95% of the D-glucose value, respectively).

Retention times of authentic PAAN derivatives are listed in Table 13 and a typical chromatogram of the standards, obtained under the conditions stated, is indicated in fig. 25. D-glucosamine was derivatised under identical conditions and applied to the column, but no material was eluted within 1h at 195°C. Seymour et al., (1979) report a retention time of approximately 30 min. for the glucosamine derivative but a temperature gradient was employed. It is therefore likely that a temperature higher than 195°C is required for elution of glucosamine PAAN derivatives.

Composition of the lipopolysaccharide extracted from each Xanthomonas strain is shown in Table 14. The results clearly demon-strate a difference between the lipopolysaccharide of the parent T646 and that of mutant strains. The wild-type lipopolysaccharide

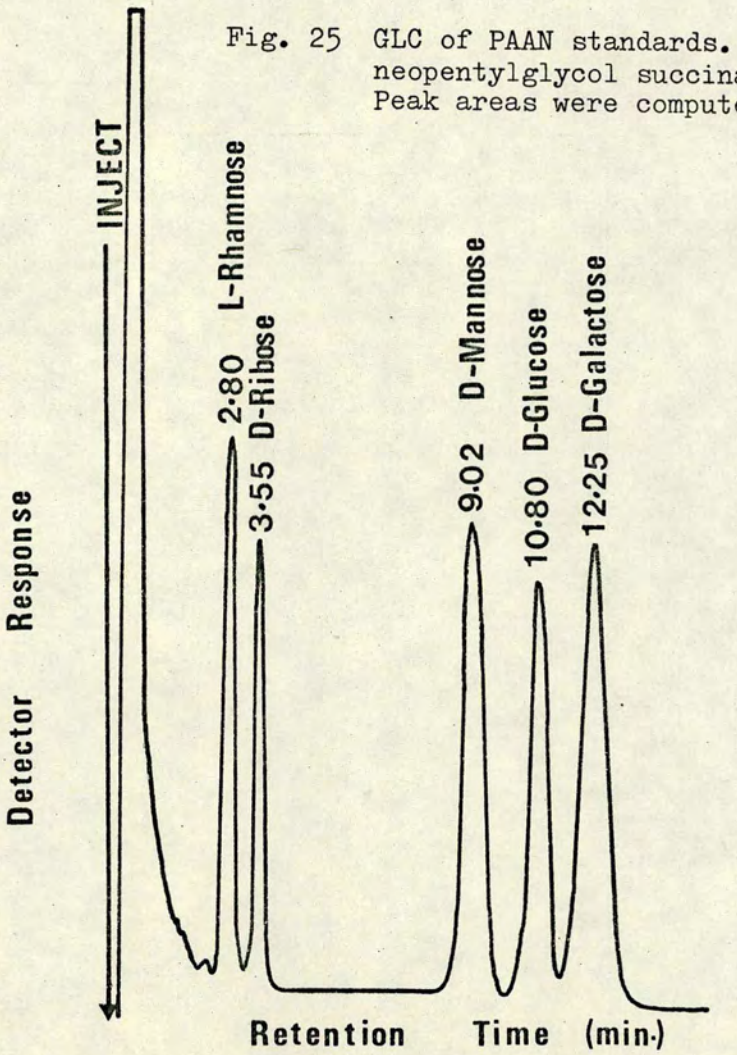


Fig. 25 GLC of PAAN standards. GLC was performed at 195°C on a column (175 x 0.3cm) of 3% neopentylglycol succinate on Chromasorb W-AW using N_2 as a carrier at 60 ml/min. Peak areas were computed using a Hewlett Packard 3380 integrator.

Table 13 Retention times for PAAN derivative standards.

PAAN DERIVATIVE	RETENTION TIME(min)
L-RHAMNOSE	2.80
D-RIBOSE	3.55
D-MANNOSE	9.02
D-GLUCOSE	10.80
D-GALACTOSE	12.25

TABLE 14. Composition of Partially Hydrolysed Lipopolysaccharide from Xanthomonas Strains.

Figures for each PAAN derivative are expressed as a percentage of the total carbohydrate; For GLC conditions see text.

STRAIN	GROWTH TEMP	PAAN DERIVATIVE				
		MANNOSE	GLUCOSE	GALACTOSE	RHAMNOSE	RIBOSE
T646	30°C	0	94.6	3.9	1.6	trace
646NM2	30°C	5.5	9.8	20.0	61.3	2.8
646D	30°C	trace	9.8	28.2	53.4	1.6
646D	37°C	0	7.5	30.6	51.0	3.1
646KR	30°C	trace	8.3	29.6	53.5	3.2
646KR	37°C	trace	5.3	31.4	52.2	0.9
646E	30°C	0	8.3	29.6	53.5	3.2
646E Soluble	30°C	4.7	8.9	27.7	53.4	1.6

was comprised almost entirely of glucose (95%), with traces of galactose (4%) and rhamnose (1.6%).

Mutants of Xanthomonas possess lipopolysaccharides of similar composition. Rhamnose (50-60%) was the major component, with lower amounts of galactose (20-30%) and glucose (5-10%). Amounts of mannose if present, were low (up to 5%). There was no difference in the lipopolysaccharides extracted from crenated strains grown at 30°and 37°C. 'Soluble' polysaccharide from 646E had apparently the same composition as the 'normal' lipopolysaccharide from mutant strains.

Traces of ribose were detected in each sample, but crude lipopolysaccharides were found to absorb at 260 nm. This result was consistent with contamination of samples with nucleic acids. It is therefore probable that the detected, and variable, levels of ribose in lipopolysaccharide hydrolysates resulted from inadequate treatment with ribonuclease during preparation.

Analysis of 646NM1 (I.W.Sutherland personal communication) indicated the presence of a lipopolysaccharide containing glucose and galactose but no rhamnose. Analysis of supernatants from 646NM1 extracts, after sedimentation of complete lipopolysaccharide, indicated the presence of rhamnose mannose glucose and galactose.

c) Exopolysaccharides.

The structure of Xanthomonas exopolysaccharide has been widely reported (see Introduction Section 7) and its repeating unit has been shown to comprise glucose, mannose and glucuronic acid in a molar ratio of 2 : 2 : 1. Pyruvate and acetate groups are variably present. The major constituents have been shown to be largely invariable under different growth conditions, although amounts of acetyl and ketal groups varied (eg Davidson, 1978; Sandford et al., (1976).

Xanthomonas campestris was grown on media supplemented with a variety of carbon sources in shake-flask cultures. Exopolysaccharide was precipitated from cell-free supernatants after 60h growth and the product analysed. Results (Table 15) show little variation in the major constituents ie glucose, mannose and glucuronic acid. Amounts

TABLE 15. Chemical Analysis of Exopolysaccharides Produced by X. campestris T646 during Growth on Different Carbon Sources.

CARBON SOURCE	% GLUCOSE	% MANNOSE	% URONIC ACID	ACETATE*	PYRUVATE*
Glucose	42	37	20	0.7	2.4
Mannose	42	40	18	0.9	2.2
Galactose	42	39	19	0.7	2.0
Xylose	39	42	19	0.7	2.0
Succinate	39	40	20	0.7	1.9
Pyruvate	41	40	19	0.9	2.8

* m mol/g polymer

TABLE 16. Hydrolytic Products of X. campestris T646 Polysaccharides after Treatment with Depolymerase H : Ratios of Major Fragments.

CARBON SOURCE	F_1**	F_2**	F_3**
Glucose	1	5.6	2.0
Mannose	1	5.1	1.8
Galactose	1	5.3	2.1
Xylose	1	5.0	1.8
Succinate	1	4.3	1.8
Pyruvate	1.	5.8	2.1

** see text.

of pyruvate varied, highest quantities were present in polysaccharides from pyruvate grown cells and lowest when succinate was the sole carbon source. Levels of acetylation were largely invariable under these conditions.

The products from cells grown in a particular media were of similar structure, irrespective of whether the polymer was harvested in early logarithmic phase, mid or late log phase or during stationary phase.

Some of the polymer samples contained traces of galactose, possibly as a result of contamination from lipopolysaccharide.

At a relatively quick method of screening exopolysaccharides from various strains, crude xanthan depolymerase from a laboratory isolate 'H' (a gift of Dr. R.E.Cripps) was used. Products of enzyme digestion varied with slight changes in xanthan structure and thus an altered pattern of fragments indicated chemical differences. The enzyme preparation contains a variety of enzyme activities (R.E.Cripps, personal communication) and is specific for xanthan or structurally similar polysaccharides. Detailed characterisation of degradation products is still in progress.

Samples (1-2mg) of exopolysaccharide were dissolved in 100μl distilled water and 50μl of crude enzyme added. Hydrolysis was carried out at 30°C for 24h ; a loss of viscosity indicated activity. Unhydrolysed material was removed by precipitation with acetone and hydrolysates reduced in volume by rotary evaporation, before being applied to Whatman 3MM paper.

Electrophoresis at 95-100mA for 4h separated three major frag--ments of carbohydrate material:- F1 ;

F_1 ; immobile on electrophoresis as were neutral sugar standards

F_2 ; acidic, with m_{glcA} of approximately 0.5

F_3 ; acidic with m_{glcA} of approximately 1.0

Polymers produced by cells grown on different carbon sources with essentially similar chemical structures (Table 15) were sub--jected to analysis with deploymerase 'H'. The fragments F_1, F_2 and F_3 were eluted after electrophoresis, pooled and aliquots analysed for total carbohydrate. The ratios of the three peaks are shown in

Table 16. Little difference is observed in polymers of similar chemical composition, as expected.

Purified fractions of F_1, F_2 and F_3 were further analysed by chromatography in solvents A, B and C respectively;

(i) Fraction F_1 gave a complex mixture of products with R_{glc} 0.1, 0.26, 0.38, 0.68, 0.81, 1.00, 1.10, 1.40 and 1.68, respectively. The major components however were R_{glc} 1.00 (glucose) R_{glc} 1.10 (mannose), R_{glc} 1.68 (acetyl-mannose).

(ii) Fraction F_2 also gave a mixture of products, the major of which had R_{glc} 0.10, 0.20 and 0.50 respectively. The nature of these products has not been precisely established, but this fraction is thought to contain the complete repeating unit and a charged tetrasaccharide.

(iii) Fraction F_3 contained one major product with the chromatographic properties of pyruvylated-mannose.

Polymers synthesised by crenated mutants, grown at 30°C on glucose, were harvested and hydrolysed with depolymerase H. Small amounts of polysaccharide were obtained from 646NM2 and the crenated mutants grown at 37°C, for analysis. In addition, the exopolysaccharide produced by strain 1128 originally classified as X. phaseoli but now falling within the heterogeneous X. campestris species (Buchanan and Gibbons, 1974) was studied. Chemical analysis of 1128 polymer indicated an authentic xanthan composition but with the absence of detectable amounts of pyruvate.

The results for the ratio of $F_1 : F_2 : F_3$ fragments from these polymers are shown in Table 17.

Products of digestion of 646NM2 indicated a similarity to the T646 product. In contrast, the products of 1128 showed a marked difference in profile. Little carbohydrate was detected in the F_3 fraction, which would be expected if no pyruvate was present; in addition the non-pyruvylated mannose released from this polymer might increase the percentage that F_1 forms of the total.

Polysaccharides produced by crenated mutants of Xanthomonas were also distinctly different to the wild-type, with ratios of fragments similar to those obtained with the non-pyruvylated 1128 polysaccharide. The purified F_1, F_2 and F_3 fractions were subjected

TABLE 17. Products of Hydrolysis of Xanthomonas Polysaccharides with Depolymerase H : Ratios of Major Fragments.

		F_1		F_2		F_3
646NM2	30°C	1	:	5.3	:	1.7
646D	30	1	:	1.4	:	0.5
	37	1	:	2.4	:	0.6
646KR	30	1	:	1.7	:	0.3
	37	1	:	1.8	:	0.4
646E	30	1	:	2.1	:	0.3
1128	30	1	:	1.6	:	0.8
T646	30	1	:	5.6	:	2.0

to chromatography in Solvents A, B and C as described and the results are shown in Table 18. These results strengthen the similarity between 646NM2 and the parental strain T646. The results for crenated mutants indicate that these strains produce a polysaccharide of similar gross composition. The composition of crenated polysaccharides appears to differ in three major areas (i) in the apparent absence of, or reduction in pyruvate (ii) the apparent absence of acetyl groups and (iii) a reduction in the amount of free mannose or an increase in free glucose in F_1.

An interesting observation with the crenated polysaccharides was that significant amounts of material remained unhydrolysed after repeated treatments with depolymerase H. Significant amounts of polymeric material also remain after treatment of T646 but in much lower quantities. Further analysis following acid hydrolysis showed the enzymically undegraded material from crenated strains to be rich in sugars found normally in lipopolysaccharide, ie galactose and rhamnose. It would therefore appear that significant amounts of lipopolysaccharide material were being released into the environ--ment either by active excretion or loss of surface material during growth, or alternatively, by overharsh treatment of cell and polysaccharide suspensions during harvesting from enamel trays. Formalin was added to these suspensions in order to 'fix' the cells.

In keeping with these observations, acid hydrolysis of poly--saccharide preparations revealed the presence of significant amounts of rhamnose and galactose detected by chromatography in Solvent A. Analysis of different preparations indicated a different composition of each. Thus the precise composition of crenated exopolysaccharide could not be established by chemical analysis. The composition of typical samples of crenated polysaccharides, obtained by the use of chemical techniques and GLC of PAAN derivatives, are shown in Table 19. As predicted by data obtained using depolymerase H, acetate and pyruvate were detected in only trace amounts.

Analysis of polysaccharide from 646NM2 confirmed its chemical structure as being authentic xanthan, with wild-type levels of pyruvate

TABLE 18. Analysis of Electrophoretic Fragments of Xanthomonas Polysaccharides by Paper Chromatography.

POLYMER		F_1 SOLVENT A			F_2 SOLVENT B			F_3
		Mannose	Glucose	O-acetyl-Mannose	R_{glc} 0.1	R_{glc} 0.2	R_{glc} 0.5	
T646	30°C	++	++	+	++	+	+	+
646NM2	30°C	++	++	+	++	+	+	+
646D	30°C	+	++	0	trace	trace	0	trace
646KR	30°C	+	++	0	trace	trace	0	trace
646E	30°C	+	++	0	trace	trace	0	trace
1128	30°C	++	+	+	trace	trace	0	trace

and acetate. Polysaccharide from 1128 showed identical chemical structure to the exopolymer from T646, but containing no detectable pyruvate (Table 19).

TABLE 19. Chemical Analysis of Exopolysaccharides from Xanthomonas Strains.

These results were obtained using two techniques. Glucose, Mannose, Galactose and Rhamnose were estimated using the GLC procedure used for lipopolysaccharide analysis. Uronic acid, pyruvate and acetate were estimated chemically.

STRAIN	% GLUCOSE	% MANNOSE	% URONIC ACID	% GALACTOSE	% RHAMNOSE	PYRUVATE*	ACETATE *
T646	42	38	20	0	0	2.4	0.7
646NM2	40	38	22	0	0	1.9	0.8
1128	42	37	21	0	0	0	0.6
646D	22	19	13	24	22	< 0.1	0.1
646E	37	12	9	28	14	< 0.1	0.1
646KR	29	21	11	27	12	< 0.1	0

* m mol/g polymer

SECTION 4. Sugar Nucleotide Biosynthesis in Xanthomonas Strains.

a) Enzymes Involved in Sugar Nucleotide Biosynthesis.

The synthesis of exopolysaccharide by Xanthomonas sp. is dependent, at least in part, upon the availability of precursors capable of donating the monomeric constituents ie glucose, mannose and glucuronic acid. Studies with other bacterial polysaccharide synthesising systems suggest that the probable precursors would be the sugar nucleotides UDP-D-glucose, GDP-D-mannose and UDP-D--glucuronic acid.

The individual enzymes involved in the synthesis of UDP-glucose, GDP-mannose and UDP-glucuronic acid (fig.26) were studied in crude extracts prepared by centrifugation (7,000g for 15 mins x 2) of an ultrasonic lysate. Both supernatant and particulate fractions were examined ; most of the enzyme activity was found to reside in particulate material.

The carbon sources glucose, gluconate citrate and glycerol support variable degrees of growth and polymer production; enzyme levels were studied in cell-free extracts prepared from cells grown in media containing these carbon sources (Table 20). Growth on glycerol resulted in a marked reduction in the amounts of hexokinase, phosphoglucomutase and UDP-glucose pyrophosphorylase, the enzymes involved in UDP-glucose synthesis and a less marked reduction in the enzymes forming GDP-mannose. Growth on citrate produced a similar trend. The amounts of biosynthetic enzymes detected in glucose and gluconate grown cells were quantitatively similar. No activity of UDP-glucose dehydrogenase was detected in any of the extracts under the assay conditions employed.

Several Xanthomonas strains, 646D, 646E, 646KR, 646NM1 and 646NM2 were grown in minimal medium containing D-glucose as sole carbon source and the amounts of biosynthetic enzymes measured (Table 21). The amounts of enzymes involved in UDP-glucose synthesis were significantly higher in the crenated mutants 646D, 646E and 646KR than those in T646 when grown under the same conditions.

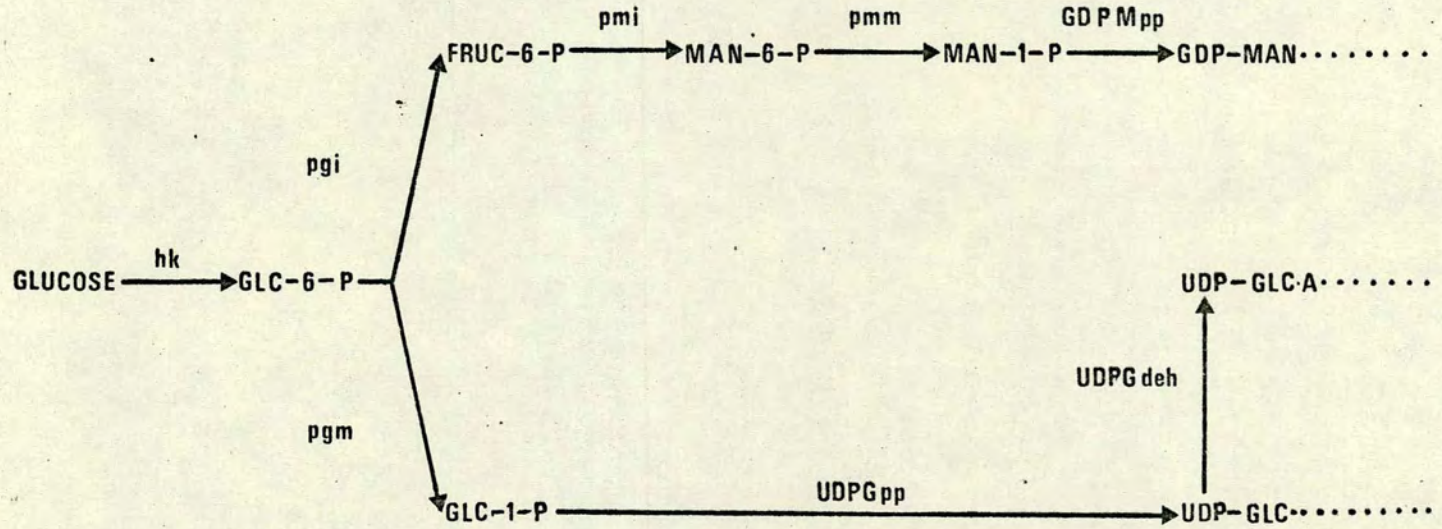


Fig. 26 The synthesis and interconversion of nucleotide diphosphate sugars. hk, hexokinase ; pgi, phosphoglucose isomerase ; pmm, phosphomannomutase ; pmi, phosphomannose isomerase ; GDPMpp & UDPGpp, sugar nucleotide pyrophosphosphorylases ; pgm, phosphoglucomutase ; UDPG deh, UDPGlc dehydrogenase.

TABLE 20. Effect of Growth Substrate on the Specific Activities of Sugar Nucleotide Synthesising Enzymes in Xanthomonas campestris T646.

ENZYME	SPECIFIC ACTIVITY μmol/min per mg protein			
	Glucose	Gluconate	Citrate	Glycerol
Hexokinase	23.0	26.1	20.3	17.5
Phosphoglucose isomerase	517	458	451	357
Phosphomannose isomerase	9.0	15.6	13.7	10.4
GDP mannose pyrophosphorylase	240	307	158	159
Phosphoglucomutase	23.5	27.5	16.8	9.5
UDP glucose pyrophosphorylase	3.1	2.9	2.6	1.2
UDP glucose dehydrogenase	n.d	n.d	n.d	n.d
Degree of Polymer Production	+++	+++	+	++

n.d not detected

Values given are the average of 2 determinations

TABLE 21. Specific Actives of Sugar Nucleotide Synthesising Enzymes in Xanthomonas Strains.

ENZYME	SPECIFIC ACTIVITY μmol/min per mg protein					
	T646	646NM1	646NM2	646D	646E	646KR
Hexokinase	23.0	37.3	21.3	17.9	15.9	4.6
Phosphoglucose isomerase	517	590	673	637	721	848
Phosphomannose isomerase	9.0	6.2	3.2	60.2	58.4	51.1
GDP mannose pyrophosphorylase	240	247	71.1	253	180.3	44.2
Phosphoglucomutase	23.5	23.2	25.5	172	195	212
UDP glucose pyrophosphorylase	3.1	1.5	0.9	7.2	6.3	6.0
UDP glucose dehydrogenase	n.d	n.d	n.d	n.d	n.d	n.d
Polymer production	+++	0	0	++	++	++

n.d not detected

Values given are the average of 2 determinations

The amounts of phosphomannose isomerase were also increased; amounts of GDP-mannose pyrophosphorylase were however comparable with the wild-type T646.

Qualitatively, there was no difference in the biosynthetic enzymes of the non-mucoid straing 646NM1 and 646NM2 and those of the wild-type T646 which would account for the lack of polymer synthesis in these strains. Quantitatively, these non-mucoid strains showed a reduction in UDP-glucose pyrophosphorylase and 646NM2 also showed a slight reduction in the amounts of the GDP-mannose synthesising enzymes when compared with T646. Whether the observed changes in enzyme activity could account for the non-mucoid phenotype in 646NM1 and 646NM2 remains unclear.

No activity of UDP-glucose dehydrogenase was detected in any of the strains studied.

The figures for enzyme activity in these strains are difficult to interpret due to the obvious differences in growth rate between strains and also in the same strain grown on different carbon sources. Activities of GDP-mannose pyrophosphorylase are particularly difficult to assess since the enzyme assay system is dependent upon no fewer than three enzymic steps. Background, increases in E_{340} for this enzyme assay were normally high. Additionally, the presence of significant quantities of particulate material in the crude enzyme preparation caused considerable fluctuation in the E_{340}. High speed supernates (200,000g for 30 mins) were therefore employed to obtain the values given. These preparations partially eliminated the problems of background activity whilst retaining sufficient enzyme activity for analysis.

b) Nucleotide Pool Analysis.

Despite the problems in comparison of enzyme levels, the detection or non-detection of enzyme activity is significant. Thus the fact that no UDP-glucose dehydrogenase activity was detected in any extract under these conditions, presented a question over the presumed role of UDP-glucuronic acid, as the precursor of glucuronic acid in exopolysaccharide biosynthesis in these bacteria. Variation of pH or cofactor requirement in the assay incubation mixture had no effect.

No evidence is available however, to indicate the possible action of a nucleotide diphosphate other than UDP, as glucuronic acid donor in bacterial exopolysaccharide. There were therefore two possible answers to this problem ; either the enzyme assay was not sensitive enough to detect enzyme activity or, a precursor other than UDP--glucuronic acid was involved. It was thought that the nature of the precursor may be established by a study of the nucleotide pool from Xanthomonas strains and in addition, such a study should confirm the evidence for the role of UDP-glucose and GDP-mannose as precursors in exopolysaccharide biosynthesis, obtained from enzyme studies.

Nucleotide pools were extracted as described in the methods section. Due to the high viscosity of culture broths, the time taken between removal of cells from the culture and ethanol extraction was probably sufficient to allow considerable change within the ethanol soluble pool. Therefore any absolute values of concentrations of purified components might not represent a true reflection of the in vivo nucleotide pools. This technique was therefore adopted as a means of characterising, but not quantifying, pool components.

Samples of the nucleotide extract were subjected to chromatography on a column of Whatman ET11 Ecteola cellulose (40 x 2cm). Eight major UV-absorbing peaks were eluted, seven of which were eluted under the gradient of triethyl ammonium acetate (fig. 27 A). Peak I : the initial uncharged peak contained the highest amount of UV absorbing material. The majority of this material was immobile on electrophoresis. A component with the electrophoretic mobility of a neutral sugar and staining with alkaline silver nitrate, was identified on rechromatography on Whatman No 1 paper in solvents A, B and C as D-glucose. This material probably remained due to inadequate washing of cells prior to ethanol extraction. The electrophoretically immobile material was tentatively identified as a mixture of nucleotides and nucleobases, by tlc in solvents D and E.

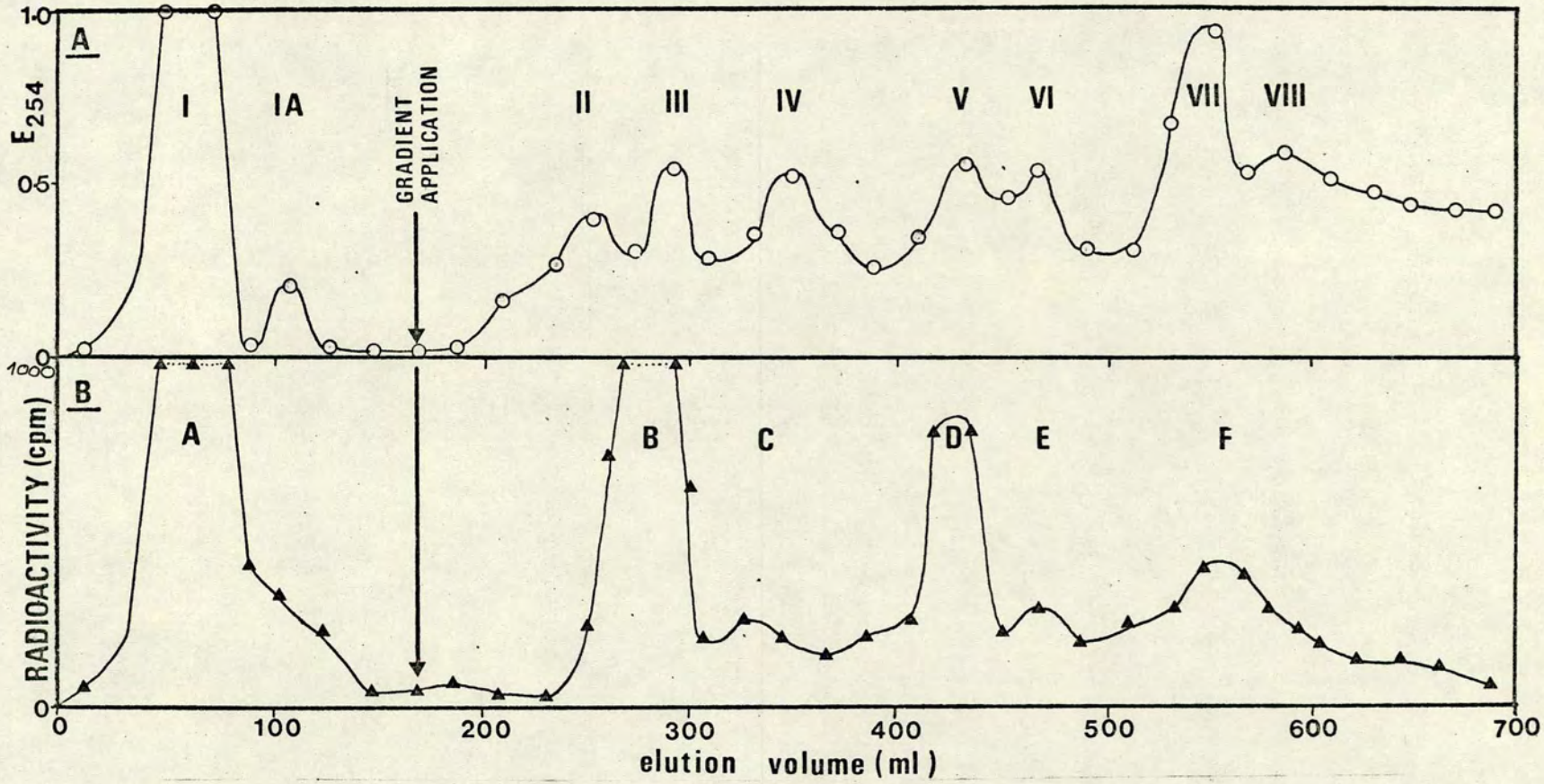


Fig. 27 Chromatography of nucleotide pools on Whatman ET11 Ecteola cellulose A T646 nucleotide extract ; B isotopically labelled [^{14}C] nucleotide pool from T646;for conditions see text.

Peak IA : a second, but not discrete, peak was also eluted prior to the application of the gradient. This material contained a mixture of components, the major of which was identified by electrophoresis and chromatography in solvents A, B and C as D-glucose. No further carbohydrate material was released from peaks I and IA by mild acid hydrolysis (0.5M H_2SO_4 for 1h).

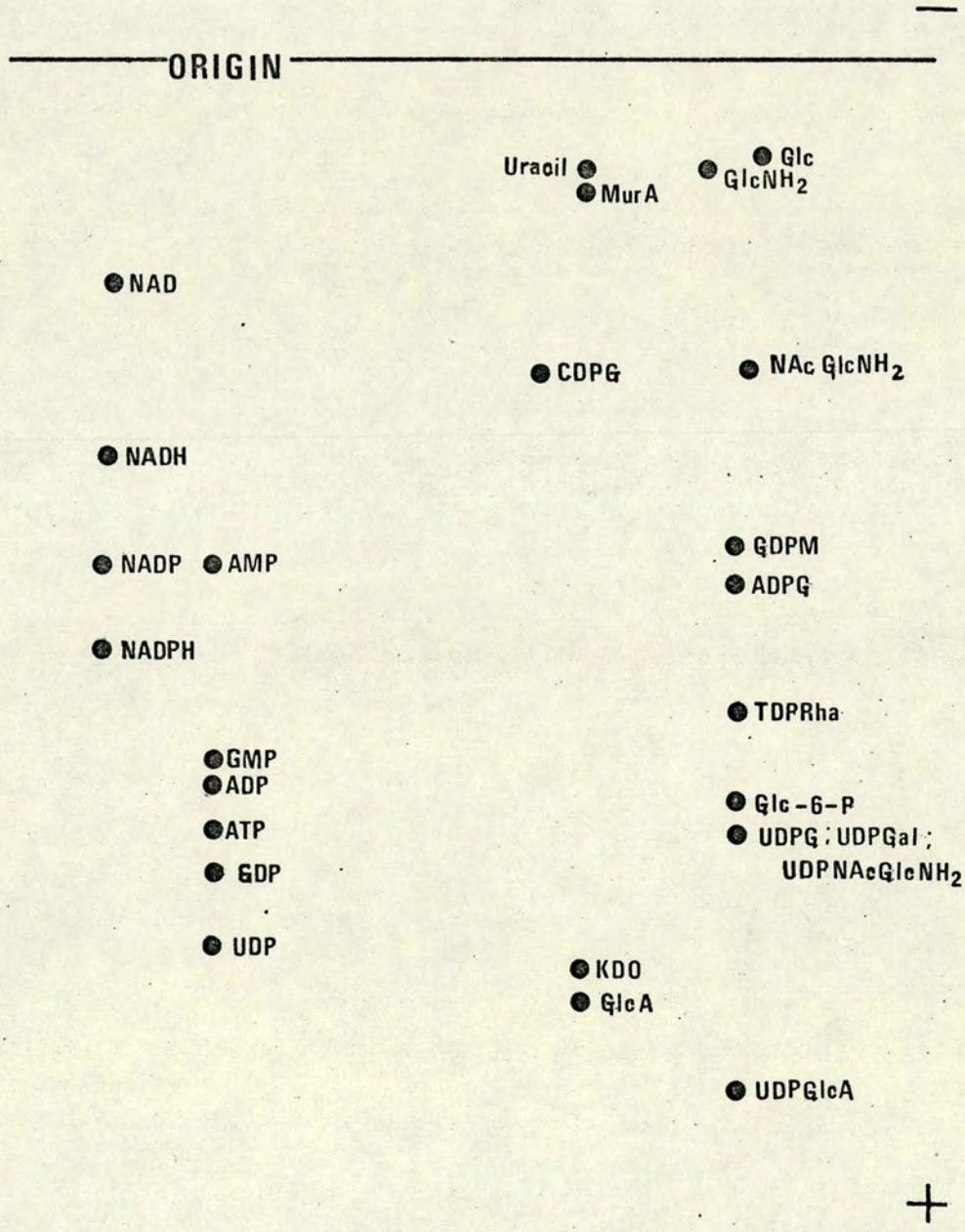
Peak II : electrophoresis followed by tlc in solvents D and E confirmed that this peak contained a mixture of components, identified as NAD^+, NADH, $NADP^+$ and NADPH when chromatographed with authentic standards. A further component which stained with alkaline silver nitrate was identified by chromatography in solvents A, B and C as 2-keto-3-deoxyoctonate (KDO). Free KDO has not been previously isolated from bacterial systems and it is probable that the acidic KDO is a breakdown product of labile CMP-KDO ; CMP was however not detected. No further carbohydrate material was released by mild acid hydrolysis (0.5M H_2SO_4 for 1h).

Peak III : this peak comprised a mixture of components with electrophoretic mobilities corresponding to UDP-glucose/UDP-galactose, GDP-mannose and trace amounts of dTDP-rhamnose. Mild acid hydrolysis (0.01M HCl for 10 mins at 100°C) released sugar material, identified by chromatography in solvents A, B and C as glucose, galactose, mannose and rhamnose. The respective nucleotides UDP, UDP, GDP and dTDP were identified by tlc in solvents D and E.

Peak IV : electrophoresis of this peak revealed a mixture of components with mobilities corresponding to a mixture of nucleotides. Rechromatography on tlc in solvents D and E confirmed the presence of AMP, ADP, UDP and GDP, with possible traces of the triphosphate derivatives, ATP, GTP and UTP. No carbohydrate material was detected prior to, or following, acid hydrolysis (0.5M H_2SO_4 for 1h).

Peak V : this peak had the electrophoretic mobility of sugar phosphates. The components were not resolved on electrophoresis. Hydrolysis (0.5M H_2SO_4 for 1h at 100°C) released neutral sugars which, on chromatography in solvents A, B and C, were identified as glucose and mannose.

Fig. 28 Electrophoresis of nucleotide standards
(4h ; 95mA ; 1.4kV; pH 5·3)



Peak VI : this peak contained a mixture of components. Several unidentified components absorbing strongly in UV; material co-chromatographing with nucleotides was released on acid hyrolysis, but no sugars were released. The unidentified components were probably a mixture of flavine nucleotides. A further component behaved similar to UDP-N-acetylglucosamine on electrophoresis; acid hydrolysis released material which stained with alkaline silver nitrate. Chromatography of the carbohydrate components in solvents A, B and C showed their identity to be glucosamine, N-acetyl glucosamine and possibly N-acetyl muramic acid. Chromatography of the nucleotide moiety using tlc in solvents D and E confirmed its identity as UDP.

Peak VII : analysis of this peak was complicated by contamination with material from peak VIII. However, electrophoresis revealed an electrophoretically mobile component which migrated with UDP--glucuronic acid. Acid hydrolysis (0.01M HCl for 10 mins at 100°C) released uronic acid, subsequently identified as glucuronic acid by paper chromatography in solvents A, B and C. The released nucleotide was subjected to tlc in solvents D and E and was identified as UDP.

Peak VIII : 'streaking' on both electrophoresis and chromatography hampered identification of this peak, but its probable identity was low molecular weight RNA and oligonucleotides. No sugar components were identified prior to, or following, acid hydrolysis (0.5M H_2SO_4 for 1h).

Due to the practical difficulties involved in collection of sufficient quantities of cells for the extraction of nucleotides, isotopically-labelled nucleotide pools were prepared from T646, 646NM2, 646D, 646E and 646KR as described in the methods section.

Six peaks of radioactivity were eluted routinely from Whatman ET11 Ecteola cellulose (fig. 27 B). On the basis of elution characteristics from the column and the identification parameters outlined above the radioactive peaks were identified as follows:-

Peak A : the initial peak was eluted prior to the application of the gradient. Only one component was identified, D-[^{14}C]-glucose, probably remaining due to inadequate washing of cells prior to extraction.

Peak B : (eluted in a similar position to peak III above) Acid hydrolysis (0.01N HCl for 10 mins at 100°C) released radioactive neutral sugars. Most of the radioactivity was in D-[^{14}C]-glucose, with smaller amounts in D-[^{14}C]-mannose and D-[^{14}C]-galactose. Trace amounts of the methyl-pentose, [^{14}C]-rhamnose were also detected.

Peak C : (eluted in a similar position to peak IV above). Only small amounts of radioactivity were eluted in this peak and its characterisation was according to elution position only; it was presumed to contain a mixture of phosphorylated nucleotides.

Peak D : (eluted in a similar position to peak V above). Electrophoresis revealed radioactive material migrating with sugar phosphates. Following hydrolysis, the majority of radioactivity remained in D-[^{14}C]-glucose with smaller amounts in D-[^{14}C]-mannose.

Peak E : (eluted in a similar position to peak VI above). Low levels of radioactivity were eluted in this peak. The major radioactive component was identified as UDP-N-acetyl[^{14}C]-glucosamine.

Peak F : (eluted in a similar position to peak VII above) contained a mixture of radioactive components; however the most active component was identified as UDP-[^{14}C]-glucuronic acid.

Six peaks of radioactivity were isolated from [^{14}C]-nucleotide extracts prepared from all the strains studied. The total radio--activity in each peak is shown in Table 22.

Peak B was composed primarily of the sugar nucleotides UDP--glucose and GDP-mannose with smaller amounts of UDP-galactose and dTDP-rhamnose. This peak contained 80-90% of the total radioactivity eluted under the gradient. Radioactivity in glucose and mannose, released on acid hydrolysis indicated a ratio of between 4.5 : 1 and 6.0 : 1 ([^{14}C]-glucose/[^{14}C]-mannose) in all the strains studied. The amounts of [^{14}C]-galactose varied widely between strains. Although significant amounts of radioactivity may be lost at each

TABLE 22. Analysis of [^{14}C]-labelled Nucleotide Extracts from Xanthomonas Strains. Figures indicate the total radioactivity (cpm) in each peak; figures in parenthesis indicate the approximate percentage of total radioactivity eluted under the gradient.

	PEAK A	PEAK B	PEAK C	PEAK D	PEAK E	PEAK F
T646	5.5×10^5	1.2×10^5 (86%)	merged with Peak B	5.1×10^3 (4%)	1.6×10^3 (1%)	7.0×10^3 (5%)
646NM2	3.4×10^5	1.3×10^5 (88%)	merged with Peak B	5.9×10^3 (4%)	2.0×10^3 (1%)	1.1×10^4 (7%)
646KR	3.2×10^5	5.1×10^5 (87%)	1.1×10^4 (2%)	5.1×10^4 (9%)	3.1×10^2 (1%)	8.1×10^3 (2%)
646D	1.5×10^5	1.9×10^5 (82%)	4.9×10^3 (2%)	2.6×10^4 (4%)	8.1×10^2 (1%)	8.1×10^3 (3%)
646E	4.1×10^5	1.5×10^5 (81%)	8.3×10^3 (2%)	3.7×10^4 (1%)	5.3×10^2 (2%)	6.9×10^3 (4%)

preparative step ie electrophoresis, elution, hydrolysis and rechromatography, the apparent similarity in glucose/mannose ratios is perhaps significant.

The sugar nucleotide UDP-glucuronic acid was isolated from the unlabelled nucleotide pool of T646 and from the isotopically labelled pools of all the strains studied. No other glucuronic acid containing sugar nucleotide was isolated from any of these strains.

The isolation of UDP-glucuronic acid tends to suggest that the assay for UDP-glucose dehydrogenase was insensitive, or alternatively, as substrate was supplied, it was immediately converted into a form against which, UDP-glucose dehydrogenase could not act. One example of this phenomenon may be in the epimerisation of UDP-glucose to UDP-galactose. The presence of enzymes capable of hydrolysing sugar nucleotides has been reported in other bacterial systems (see Introduction : Section 7); the release of free $[^{14}C]$-glucose from UDP $[^{14}C]$-glucose, was catalysed by a crude membrane preparation from Xanthomonas T646. It is unlikely however that UDP-glucuronic acid was synthesised by a route other than from UDP-glucose.

Isotopically labelled nucleotide pools were extracted from cells in non-growing washed cell suspensions. As a result, precursors involved in the synthesis of the structural polysaccharides, peptidoglycan and lipopolysaccharide eg UDP-N-acetyl glucosamine (Peak E); dTDP-rhamnose and UDP-galactose (Peak B) were found at lower levels than those precursors involved in exopolysaccharide synthesis.

SECTION 5. Exopolysaccharide Synthesis by Whole Cells.

a) Washed Cell Polymer Synthesis.

Growth and polymer production by cells grown on glucose would indicate that the monosaccharide components of exopolysaccharide could be synthesised from D-[^{14}C]-glucose. Since under normal growth conditions [^{14}C]-label would be incorporated into most cellular components, washed cell suspensions were used for whole cell biosynthesis studies. The absence of a nitrogen source effectively prevents growth and much of the [^{14}C]-glucose might be expected to be utilised in exopolysaccharide synthesis.

Cells were harvested after 16h growth in YE medium containing 2% D-glucose, washed twice and finally resuspended in YE salts (200mg wet cells/ml final concentration). Radio-labelled [^{14}C]--glucose (3mCi/m mol) was added at a level of 0.5μCi/ml with 0.5mg/ml cold carrier glucose. Polymer synthesis was assayed in duplicate 100μl aliquots of cell-free supernatant; samples were applied to Whatman 3MM paper and irrigated overnight in solvent D, origins were then dried and counted in PPO scintillant. Incorporation of radioactivity into chloroform/methanol, 2 : 1 (CM) and chloroform/methanol/water, 10 : 10 : 3 (CMW) was assayed in 250μl aliquots of incubation suspension as described in the methods section.

Radioactivity was incorporated from [^{14}C]-glucose into polymer and solvent extractable material by X. campestris T646 cells over a 2h time course (fig. 29 A). After 2h, cells were deposited by centrifugation and the decanted supernatant dialysed against running water to remove free [^{14}C]-glucose, before freeze-drying. The material was subjected to acid hydrolysis followed by chromatography in solvents A and C. Radioactivity, detected by counting cut segments of the chromatogram, migrated with Rfs corresponding to glucose (57% of the total radioactivity), mannose (34%) and glucuronic acid (9%).

Incorporation of [^{14}C]-mannose into polymer and solvent extractable material occurred at a lower level in glucose-grown Xanthomonas T646 (fig. 29 B). Insufficient polymer was produced

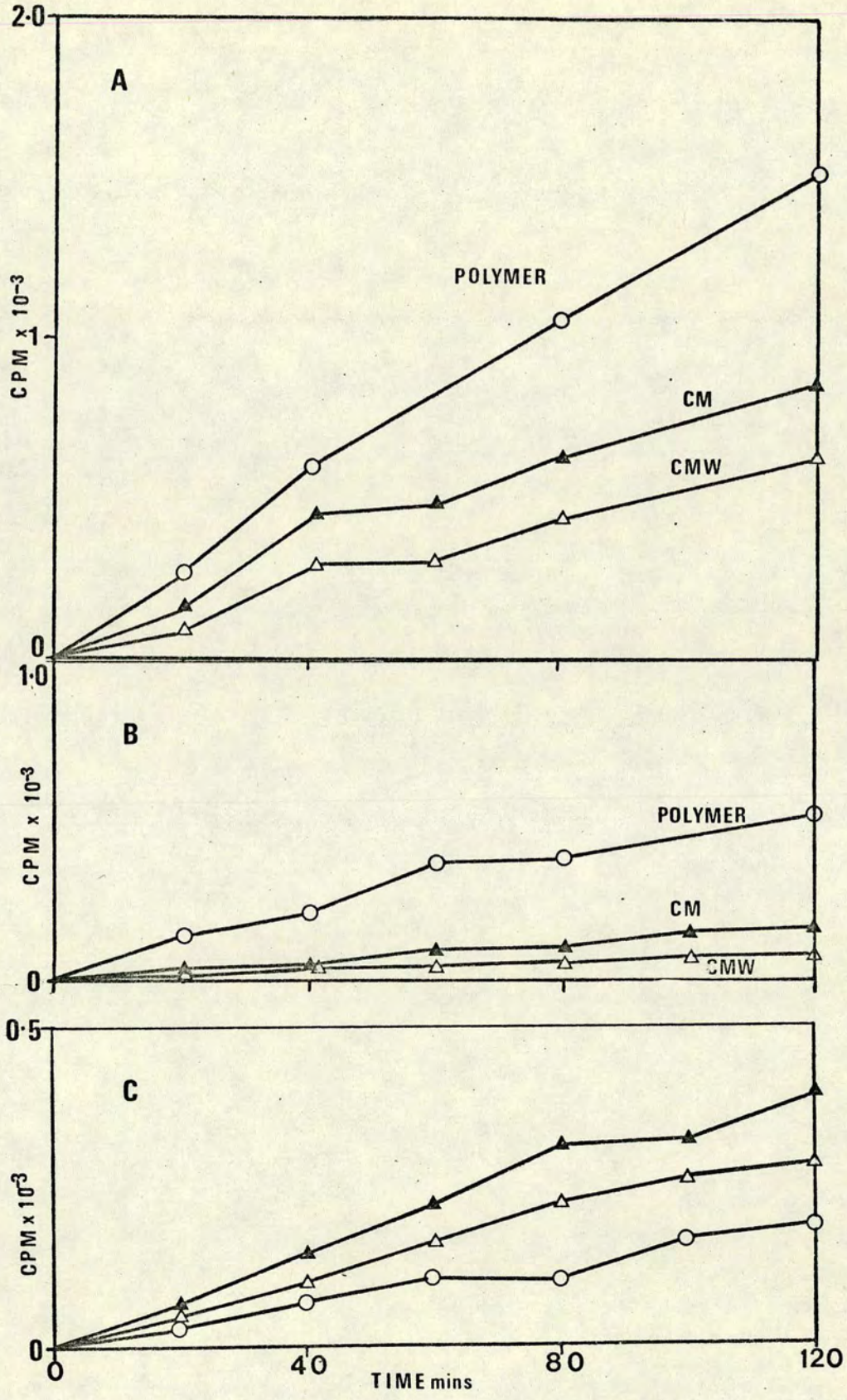


Fig. 29 Incorporation of label from [^{14}C]hexose into polymer(○), CM (▲) and CMW (△).
A T646C cells incubated with [^{14}C]D-glucose
B T646 cells incubated with [^{14}C]D-mannose
C 646E cells incubated with [^{14}C]D-glucose

for adequate analysis.

Synthesis of exopolysaccharide by washed cell suspensions of Xanthomonas crenated mutants was investigated. Similar results were obtained with each strain. 646D, 646E and 646KR; fig. 29 C shows results from 646E. Lower amounts of radioactivity were incorporated into polymer (3-6% of wide type levels) and solvent extractable material (40-50%).

b) Labelling of polysaccharide fractions in washed cell suspensions.

In an attempt to study the fate of [^{14}C]-glucose in more detail, levels of radioactivity in exopolysaccharide, lipopolysaccharide, nucleotide pools and solvent (CM) extracts were analysed in washed cells. After 60 min. incubation, cells were harvested from the incubation mixture and resuspended in a small volume of distilled water. Aliquots were added to 4ml boiling ethanol and the nucleotide pool was extracted as described previously. Extracts were evaporated to dryness and counted in Toluene-Triton Scintillant. Lipopoly--saccharides were extracted in 4ml 45% aqueous-phenol at 65°C, as described previously. The dialysed lipopolysaccharide was freeze-dried and counted in Touluene-Triton scintillant.

Levels of radioactivity in each fraction of the Xanthomonas strains studied are listed in Table 23. Amounts of radioactivity in nucleotide extracts were similar in each strain. Figures for CM extracts indicated higher levels of radioactivity in T646 and 646NM2 than in the crenated strains. Amounts of radioactivity in lipopolysaccharides were comparable. As expected, highest levels of incorporation into polymer occurred in the wild-type, with only trace amounts detected in 646NM2.

Incubation of crenated strains at 37°C had little effect on amounts of radioactivity in the nucleotide pool. Values for lipopolysaccharide and CM fractions were reduced to 60% of the 30°C figures; no exopolysaccharide was synthesised.

c) The nature of CM and CMW extracts.

Incorporation of radioactivity into CM and CMW extracts during polymer synthesis perhaps indicated the involvement of lipid intermediates in that synthesis. The presence of isoprenoid alcohol

TABLE 23. Distribution of Radioactivity in Washed Cell Suspensions ; % Labelling of Fractions.

STRAIN	INCUBATION TEMPERATURE (°C)	NUCLEOTIDE EXTRACT	CM EXTRACT	LIPOPOLYSACCHARIDE	EXOPOLYSACCHARIDE
T646	30	5.1	1.1	2.2	2.3
646NM2	30	5.1	0.9	2.0	< 0.1
646D	30	5.1	0.5	2.7	0.2
646E	30	5.1	0.5	2.8	0.1
646KR	30	5.0	0.5	2.7	0.1
646D	37	5.0	0.3	1.9	< 0.1
646E	37	4.8	0.3	1.7	< 0.1
646KR	37	4.9	0.3	1.6	< 0.1

derivatives in non-saponifiable lipids, extracted from X. campestris T646, was established. Samples from the 1 : 9 diethyl ether/light petroleum fraction were subjected to tlc on silica gel G plates, irrigated in solvent F. One major spot (R_f 0.66) and a second minor spot (R_f 0.60) were visualised with iodine vapour; authentic ficaprenol migrated w th R_f 0.69 and C_{55}-isoprenol phosphate with R_f 0.62 under the same conditions. The slower moving spot stained for phosphorus with Dittmer's reagent (Dittmer et al., 1974).

Further chromatography in solvent G confirmed the identity of these fractions as being C_{55}-isoprenoid alcohol and its monophosphate derivative.

Further indication of the involvement of isoprenoid lipids in exopolypaccharide synthesis was obtained with bacitracin. The antibiotic bacitracin acts primarily on the recycling of lipid phosphates (Introduction: Section 3). Addition of bacitracin (0.5mg/ml final concentration) was inhibitory with respect to the incorporation of radioactivity from [^{14}C]-glucose into polymer and CM extracts of Xanthomonas T646 (fig. 30A). Bacitracin at 0.5mg/ml or 2.0mg/ml was ineffective in washed cell suspensions of 646E but addition of 10mg/ml bacitracin raised inhibition of polymer synthesis and prevented further incorporation into CM extracts (fig. 30B). In both strains, polymer synthesis ceased within 5 min of antibiotic addition, after which, levels of radioactivity in CM extracts declined slightly.

Incorporation of radioactivity into solvent extractable material was low in routine incubations. Optimisation of conditions showed that although incubations at 30°C and pH 7.0 were favourable, cell density was an influential factor in determining the extent of radioactive incorporation into CM and CMW (fig. 31). Subsequent incubations contained 15mg wet cells/ml (final concentration).

In attempts to obtain large amounts of CM and CMW material, pooling of material from parallel incubations was found to be more effective than use of large volume incubations. Cells were extracted three times with 4 vols. CM or CMW; as shown in Table 24, the majority of radioactivity was extracted by the second treatment. Pooled extracts were washed twice with distilled water and the lower

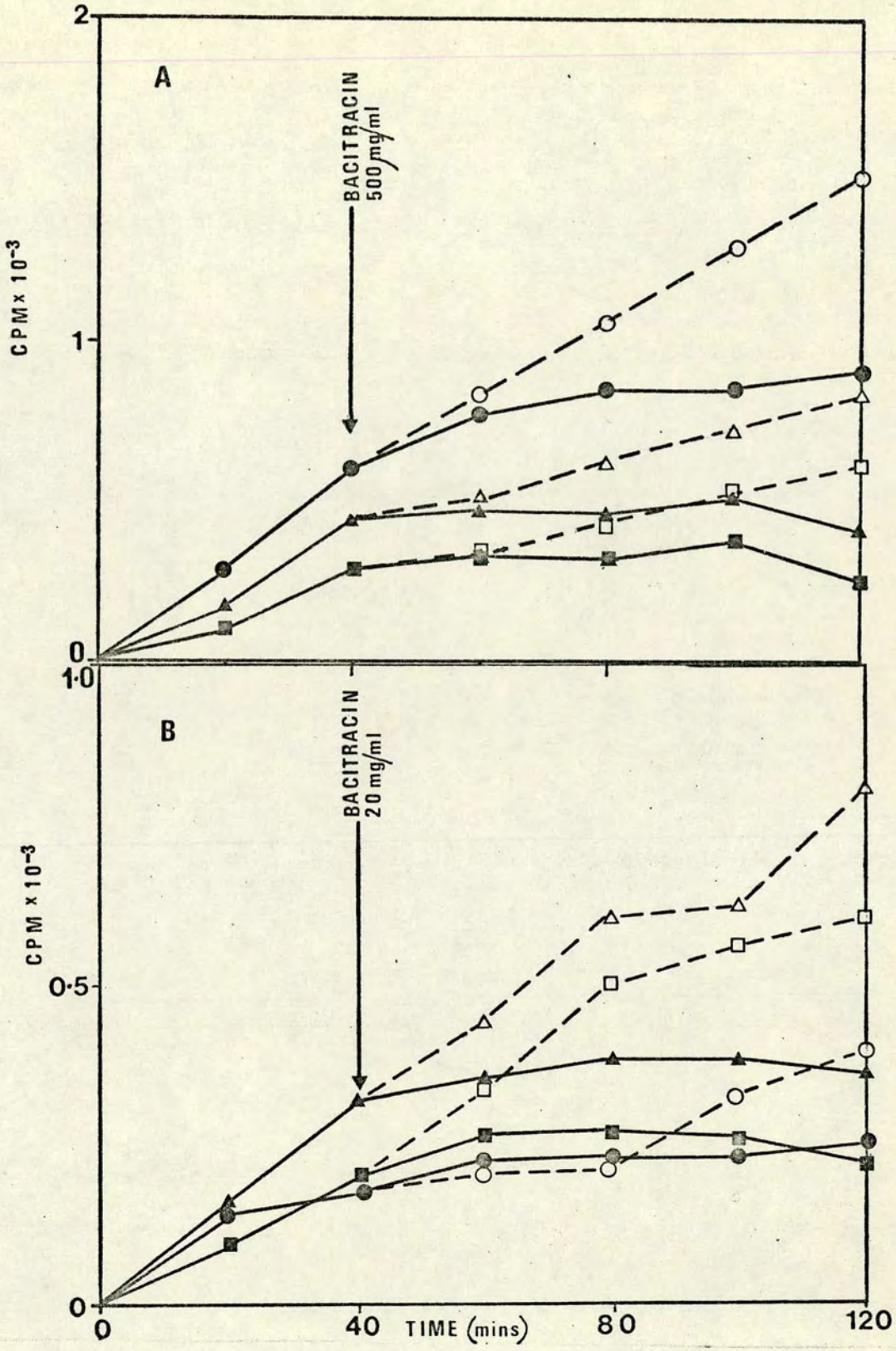


Fig. 30 Effect of bacitracin on incorporation of label from [^{14}C]-glucose into polymer (●,○) CM(▲,△) and CMW (■,□) by washed cells. Broken lines and symbols denote control incubations. A T646 + 500µg/ml bacitracin
B 646E + 20mg/ml bacitracin

TABLE 24. Effect of Multiple Extraction on the Radioactivity in CM and CMW Extracts from Xanthomonas T646

EXTRACTION	% TOTAL RADIOACTIVITY EXTRACTED	
	CM	CMW
1	24.5	42.5
2	61.0	50.0
3	14.5	7.5
4 and subsequent	0	0

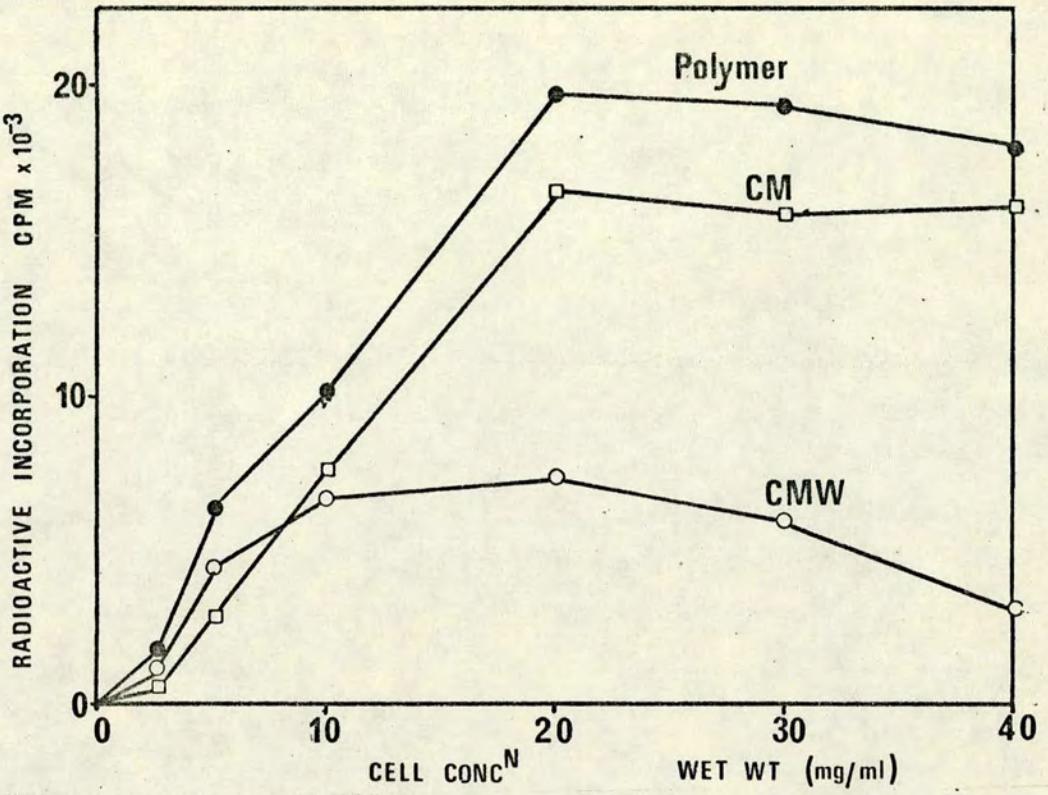


Fig. 31 Effect of cell density on incorporation of label from [^{14}C]glucose into polymer (●), CM (□) and CMW(○) by washed T646 cells

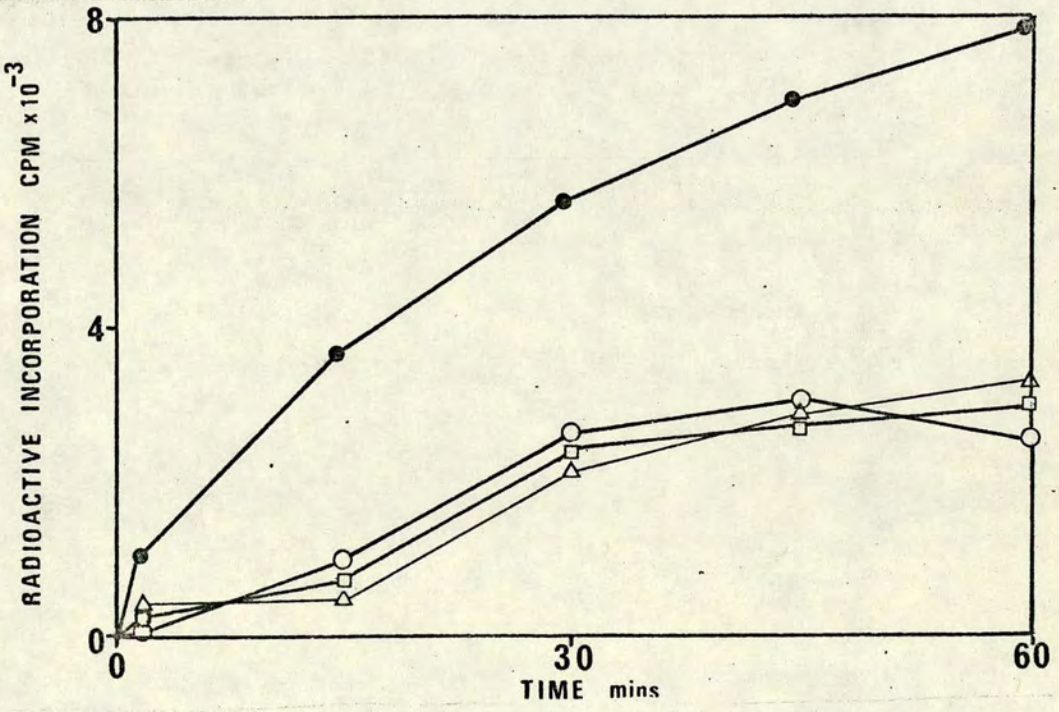


Fig. 32 Radioactive incorporation into CM extractable material, from T646 washed cells. Lipids I(●), II(△), III(○) & IV(□).

organic phase retained.

CM and CMW extracts were subjected to ion-exchange chromatography on DEAE cellulose (Whatman DE52). DEAE-cellulose was converted to the acetate form according to the method of Rouser, Kritchevsky, Yamamoto, Simon, Galli and Bauman, (1969) and packed in a glass column (6 x 0.8 cm). Lipids were eluted from the column using stepwise gradients of ammonium acetate in CM. Fractions (1.5 ml) were collected at a flow rate of 50ml/h, aliquots (50µl) were removed and counted in Toluene-Triton scintillant. Four peaks of radioactivity were eluted routinely(fig. 33). A neutral peak (Lipid I) was eluted prior to application of the gradient ; one peak (Lipid II) was eluted with 10mM ammonium acetate and a further two anionic peaks (Lipids III and IV) with 50mM ammonium acetate. No further radioactivity was eluted in increased concentrations of ammonium acetate. Similar components occurred in CM and CMW extracts and in further studies these extracts were combined.

Extracts from crenated strains prepared under similar conditions, had the same qualitative composition as the wild-type extract. Figure 33 also shows the elution profile for 646E, lipid II contained a higher percentage of the total radioactivity than its T646 equivalent and lipid IV was similarly increased,but lower levels of radioactivity occurred in lipid I.

Since the mixture of components was complex and the resulting level of radioactivity in each component low, the effect of incubation time on incorporation into each lipid was investigated. The results (fig. 32) showed that the amount of radioactivity in lipid I increased throughout a 60 min incubation, but maximum levels of lipids II, III and IV were detected after 30 min. Subsequent incubations were terminated after 30 min.

(i) Analysis of Lipid I : The neutral lipid contained more than 40% of the total radioactivity in Xanthomonas T646 CM and CMW extracts and was also the predominant peak in 646E.

Analysis by tlc on Whatman SG81 paper and silica gel G irrigated in solvent H, revealed a mixture of components with mobilities consistent with triglycerides, sterol esters and possibly

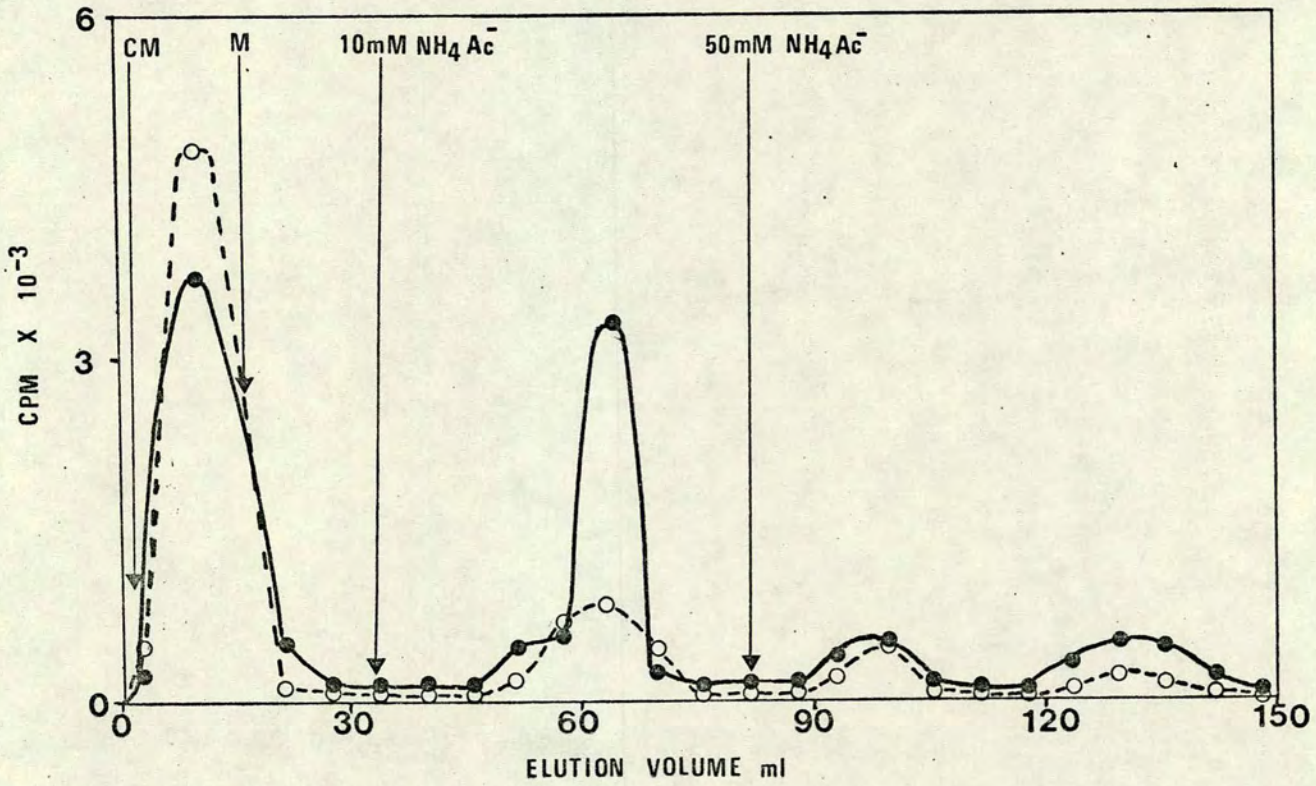


Fig. 33 Elution profile of CM extracts on DEAE cellulose. 646E extract,●—●; and T646 extract,o-o. For conditions see text.

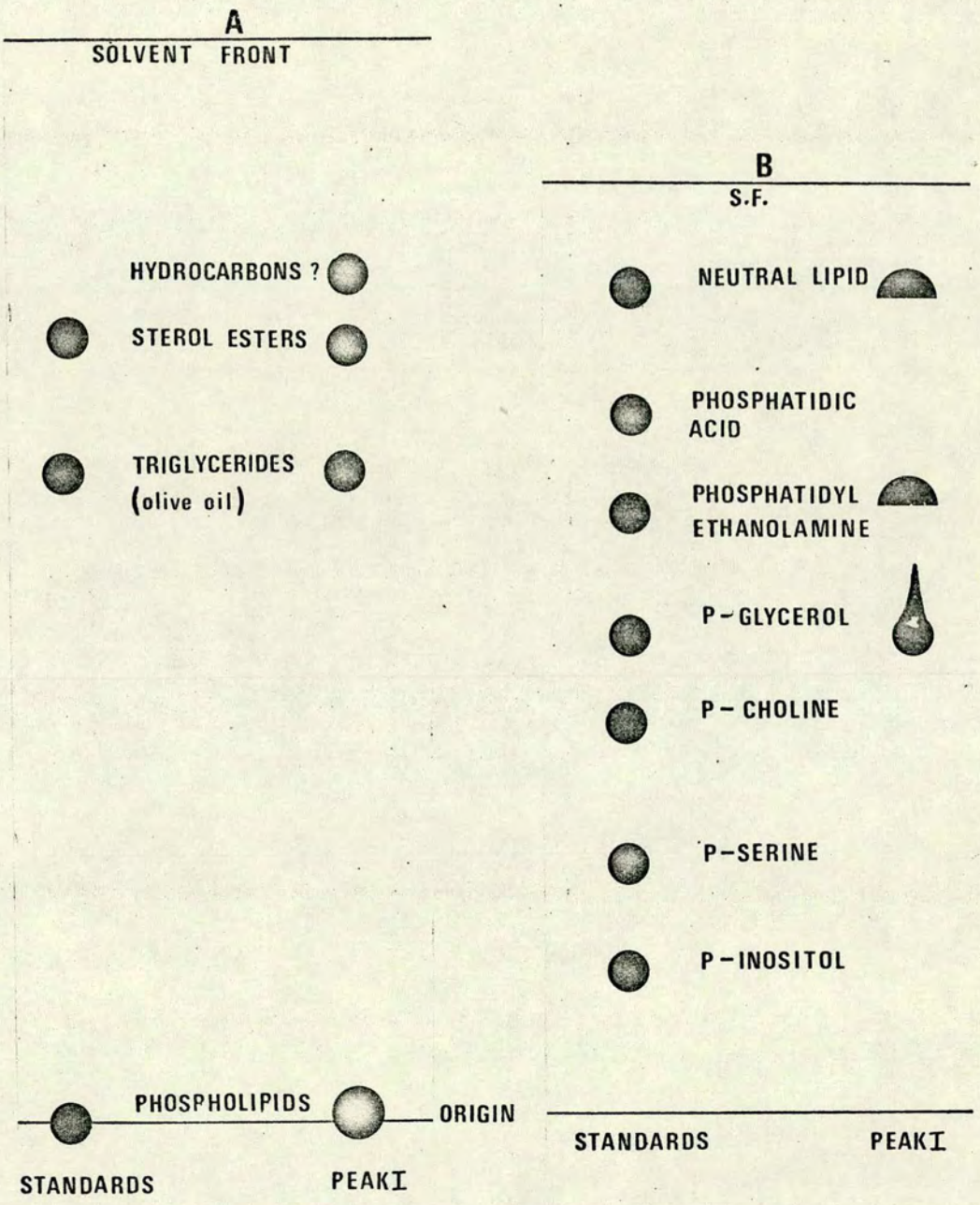


Fig. 34 Analysis of CM extract lipid I by tlc : A, Solvent H; B, Solvent J.

hydrocarbons (fig. 34A). Phospholipids were not mobile in this system. Further tlc in solvent J revealed two phosphlipids (stained with Dittmers's reagent). The major fraction had the mobility of phosphatidylglycerol/cardiolipin and as would be expected if this identify was correct, it was Schiff positive. The minor spot had a mobility similar to phosphatidylethanolamine. A further fraction co-chromatographed with neutral lipids and was stained in iodine vapour but not by Dittmer's reagent.

This peak also contained free radioactive glucose and possible decomposition products of the anionic peaks. Rechromatography of lipid I on DEAE-cellulose yielded no further separation, thus the high levels of radioactivity did not result from column overloading.

(ii) Analysis of anionic lipids II, III and IV : Anionic lipids were chromatographed on a column of Sephadex LH20 (35 x 1 cm) with 1M ammonium acetate in 99% methanol as eluant, according to the method of Dankert, Wright, Kelley and Robbins, (1966). Differences in molecular weight were reflected in differences in elution. From the results obtained (fig. 35), lipid IV had the highest molecular weight; the molecular weight of lipid III was slightly greater than that of lipid II.

The three anionic lipids were resistant to treatment with mild alkali (0.05N KOH at 37°C) (fig. 36A). However all were hydrolysed by mild acid (0.01N HCl in 25% 1-propanol at 100°C), the majority of radioactivity being released into the aqueous phase after 30 min (fig. 36B). The results were consistant with isoprenoid alcohol phosphate derivatives. Small quantities of acid-resistant material probably contained cyclic phosphate esters, formed during preparation and purification of the lipids.

Lipids III and IV were hydrolysed in 45% (w/v) phenol at 60°C for 5 min. (fig. 36C); lipid II was stable to phenol treatment. Phenol cleaves pyrophosphate linkages in isoprenyl compounds and this data, in conjunction with the molarity of ammonium acetate required for elution from DEAE-cellulose suggest that lipid II carried a monophosphate linkage but that lipids III and IV were diphosphate derivatives.

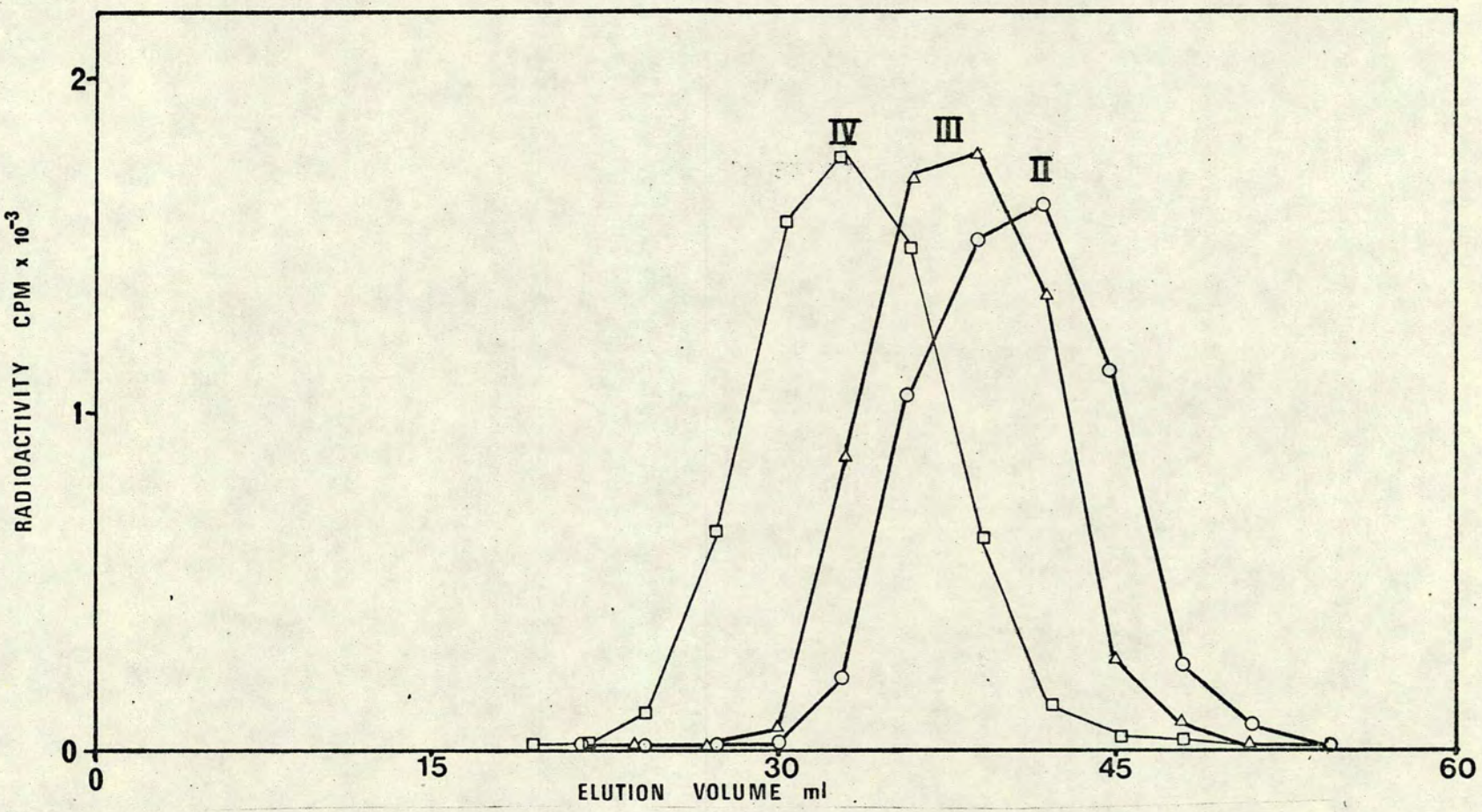


Fig. 35 Chromatography of lipids II, III & IV on Sephadex LH20. For conditions see test

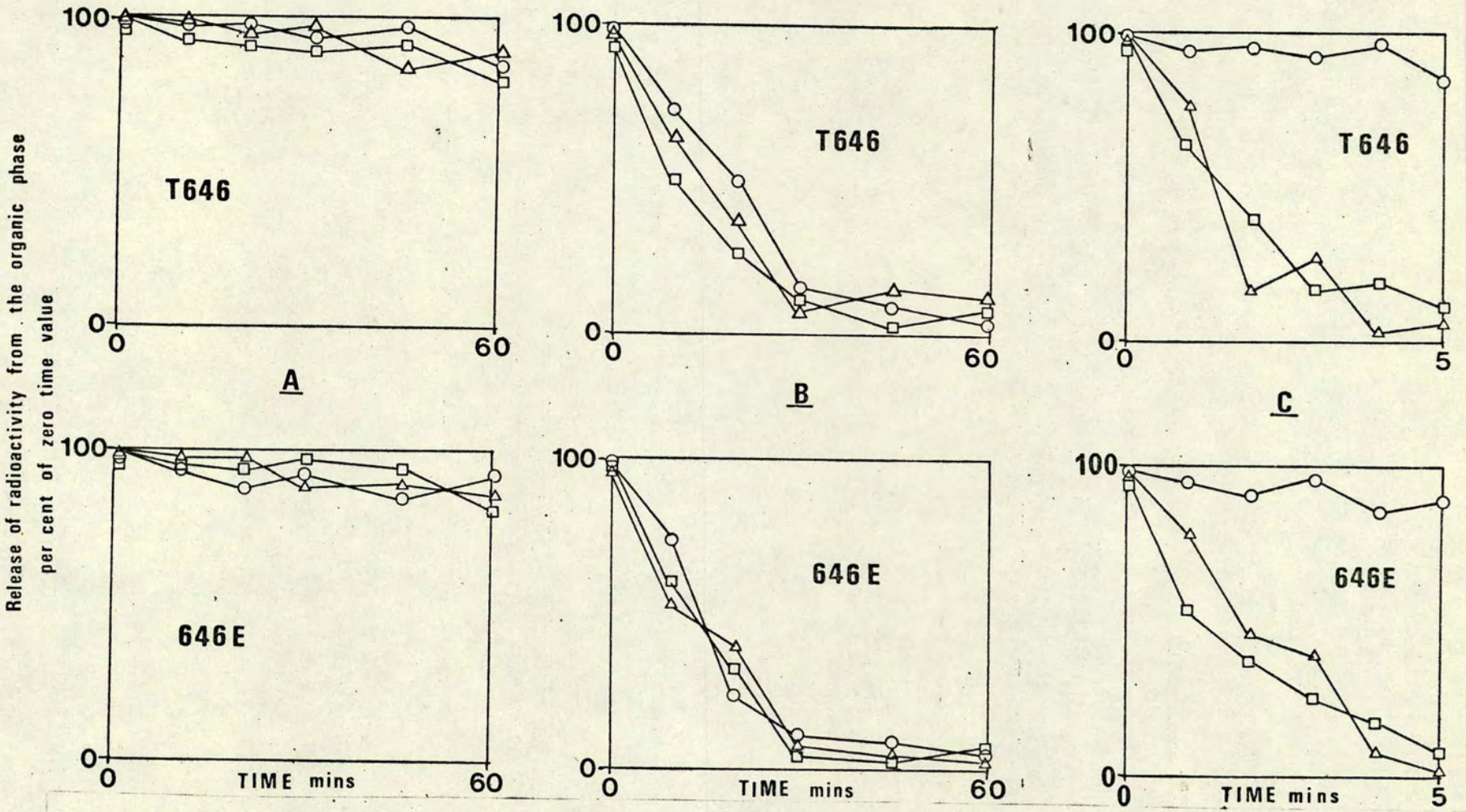


Fig. 36 Hydrolysis characteristics of lipids II (○), III (△) & IV (□). Lipids from T646 CM extract (upper frame) and 646E CM extract (lower frame) subjected to A, mild alkali (0.05N KOH, 37°C); B, mild acid (0.01N HCl in 1-propanol at 100°C) C, 45% aqueous phenol at 60°C.

Hydrolysis in 2N H_2SO_4 for 3h at 100°C released the radioactivity from anionic peaks into the aqueous phase. Neutralised material from each peak failed to move on electrophoresis. Chromatography in solvents A and C identified the neutral sugar from lipid II as being galactose ; the radioactive product from both lipids III and IV was identified as glucose.

Aqueous, radioactive products of phenol hydrolysis of lipids III and IV were phosphorylated derivatives. Treatment with commercial alkaline phosphatase for 2h at 30°C released free sugars ; under the same conditions, 1m mol of glucose-1-phosphate was completely dephosphorylated. Chromatography of free sugars obtained by this process in solvents A and C, identified the presence of glucose and a disaccharide from peaks III and IV, respectively.

The presumptive mono- and disaccharides were chromatographed on Biogel P2. A glass column (28 x 1.2 cm) was run at a flow rate of 10ml/h, using distilled water as eluant. Fractions (250µl) were collected and 50µl samples counted for radioactivity in Toluene--Triton scintillant. As expected, radioactivity chromatographing with authentic mono- and disaccharides, was detected (fig. 37).

Hydrolysis of the three anionic lipids in 40mM trifluoroacetic acid (Sandermann, 1977) released free mono- and disaccharides from lipids III and IV.

Acid hydrolysis of the disaccharide moiety released only glucose. Hydrolysis with commercial β-glucosidase indicated a cellobiosyl rather than maltosyl derivative.

The anionic lipids II, III and IV were therefore tentatively identified as isoprenyl monophosphate galactose, isoprenyl diphosphate glucose and isoprenyl diphosphate cellobiose, respectively. No intermediate of longer chain-length was extracted in either CMW or in solvents containing increased aqueous phases. No intermediates containing mannose or glucuronic acid were identified.

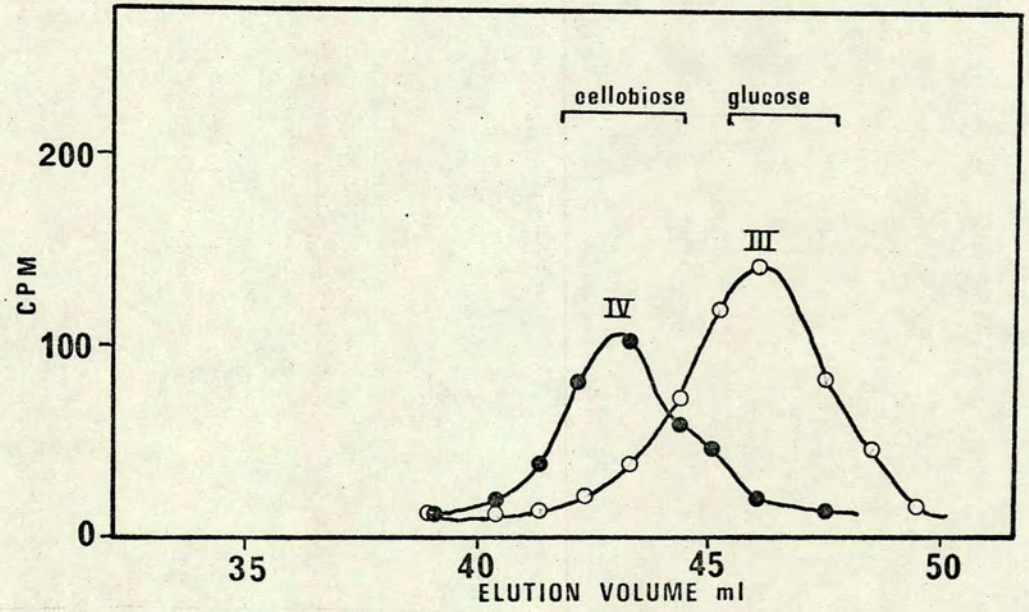


Fig. 37 Chromatography of dephosphorylated oligosaccharides from lipids III and IV on Biogel P2. For details see text.

SECTION 6. Exopolysaccharide Synthesis by Cell-Free Preparations.

a) Preliminary Studies.

A particulate, cell-free preparation was used in an attempt to study exopolysaccharide synthesis in detail and, if possible, in isolation. Membranes were prepared from cells grown in 1-litre batches of glucose broth. Cells were harvested after 16h, washed twice in distilled water and resuspended in 50mM 3-(N-Morpholino) Propane Sulphonic acid (MOPS) buffer pH 7.8. The suspension was lysed by ultrasonication with cooling on ice/ethanol. Unbroken cells and debris were removed by centrifugation (7,000g, 15 min at 0°C); membranes were sedimented and washed by further centrifugation steps (100,000g, 1h at 0-4°C).

Routine incubation mixtures comprised 50mM MOPS pH 7.8, 150μl; 100mM $MgCl_2$, 10μl; 50mM dithiothreitol, 10μl; 40mM UDP-glucose 5μl; 40mM GDP-mannose, 5μl; 20mM UDP-glucuronic acid, 5μl; 20mM PEP, 5μl; 20mM Acetyl CoA, 5μl. A final volume of 600μl routinely contained 5mg protein.

Radioactive incorporation into duplicate CM (50μl) and polymer (20μl) samples was assayed as described previously.

Radioactivity from UDP-[^{14}C]-glucose was incorporated into polymer and CM by Xanthomonas T646 membranes in the presence of all the presumed precursors of xanthan (fig. 38). Similar incorporation occurred in the presence of UDP-[^{14}C]-glucose alone (fig. 39). No significant amounts of radioactivity were incorporated from GDP-[^{14}C]-mannose into polymer or CM. Use of [^{14}C]-phosphoenol--pyruvate or UDP-[^{14}C]-glucuronic acid was procluded since these substrates were non-specifically bound to an undefined component, giving high zero-time values for incorporation into polymer : these values did not increase with subsequent incubation.

Similar results were obtained with membranes prepared from crenated strains.

It thus appeared that only UDP-glucose was incorporated into polymeric material, either directly, or indirectly following conversion into another precursor. Incorporation of radioactivity from UDP-[^{14}C]-glucose into polymer was therefore studied in detail.

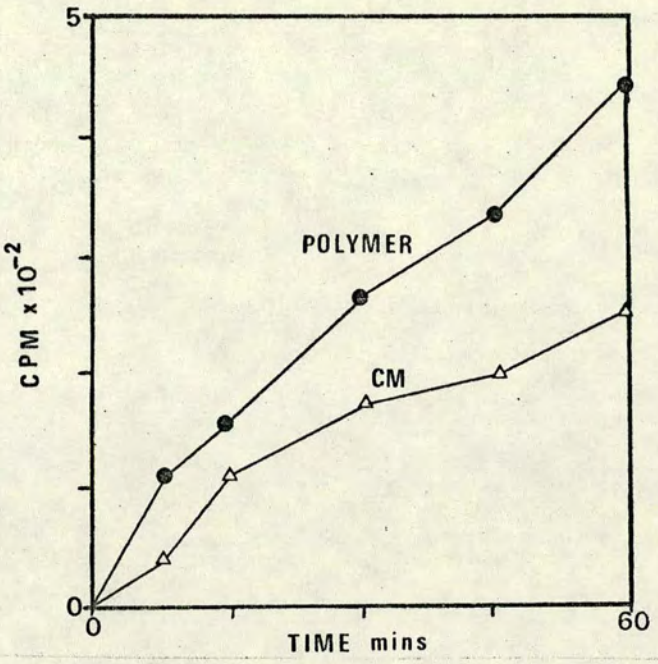


Fig. 38 Incorporation of [^{14}C] from UDP[^{14}C]Glucose into polymer in the presence of GDP-mannose, UDP--glucuronic acid, PEP & acetyl CoA, by T646 membranes

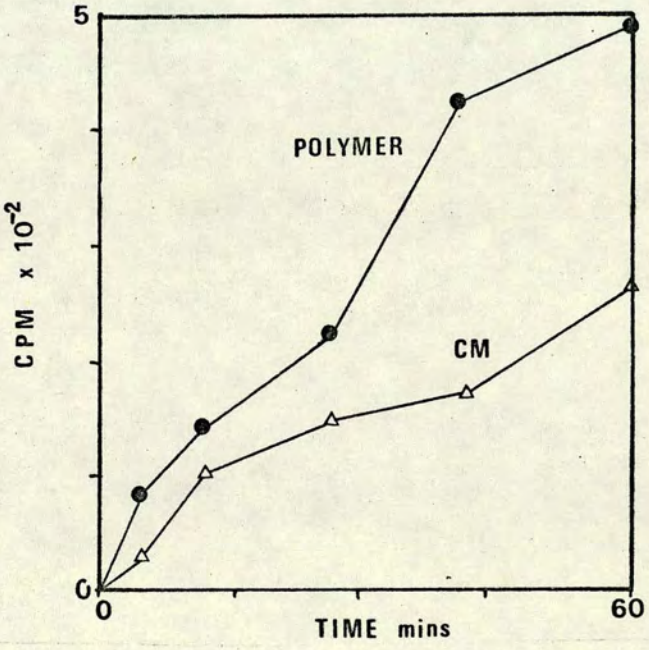


Fig. 39 Incorporation of [^{14}C] from UDP[^{14}C]-glucose into polymer by T646 membranes.

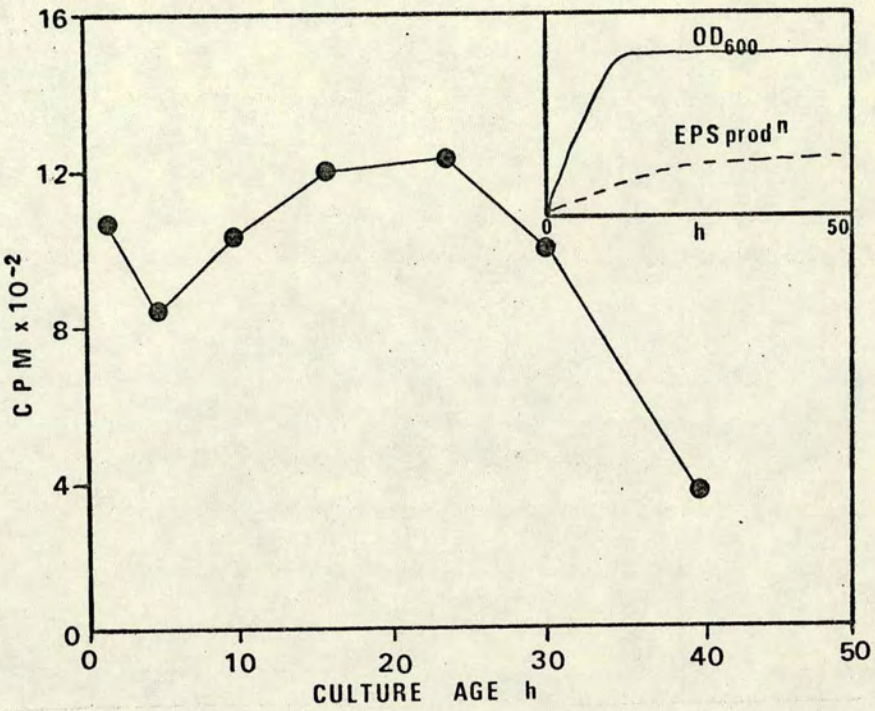


Fig. 40 Effect of culture age on [^{14}C] incorporation from UDP[^{14}C]gluccose into polymer by T646 membranes.

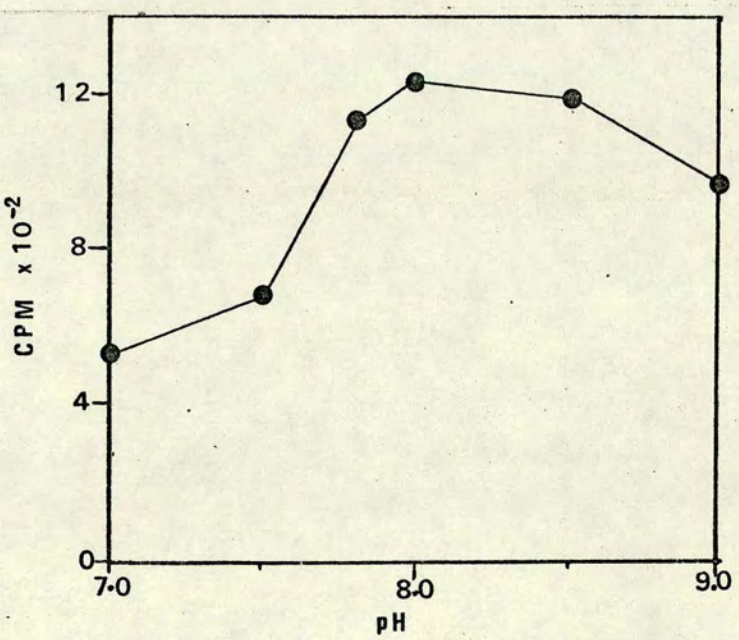


Fig. 41 Effect of pH on [^{14}C] incorporation from UDP[^{14}C]glucose into polymer by T646 membranes.

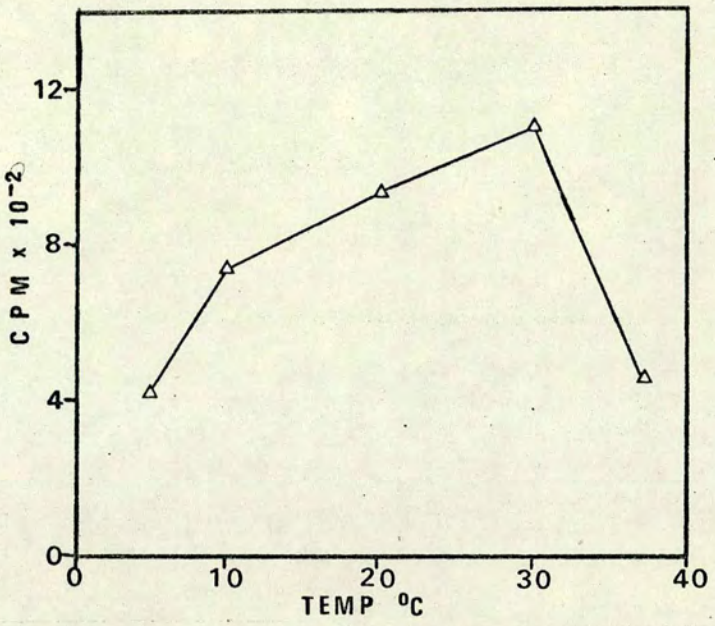


Fig. 42 Effect of temperature on [^{14}C] incorporation from UDP[^{14}C]glucose into polymer by T646 membranes.

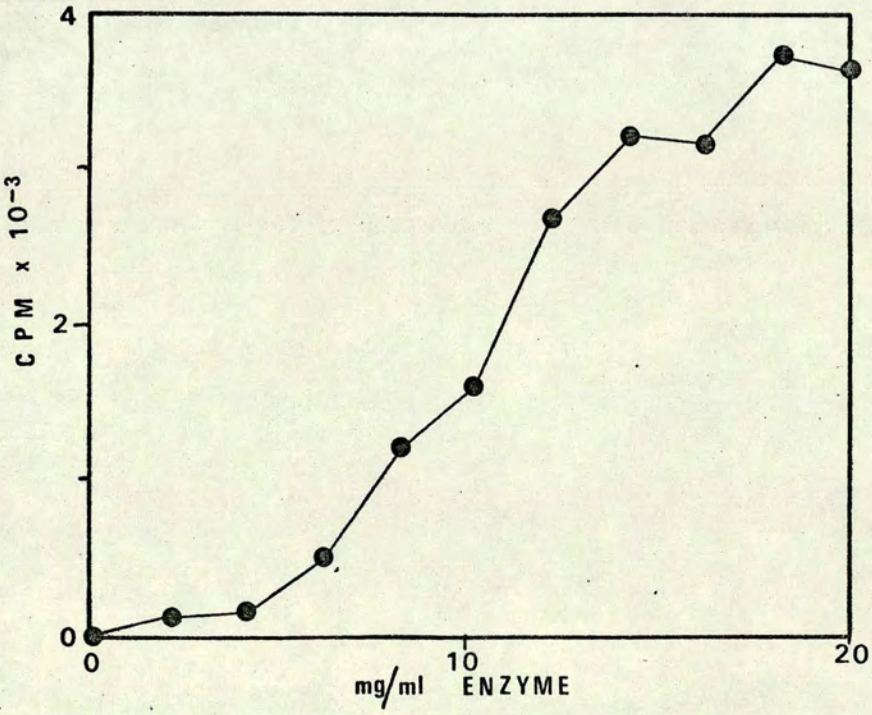


Fig. 43 Effect of enzyme conc. on [^{14}C] incorporation from UDP[^{14}C]glucose into polymer by T646 membranes.

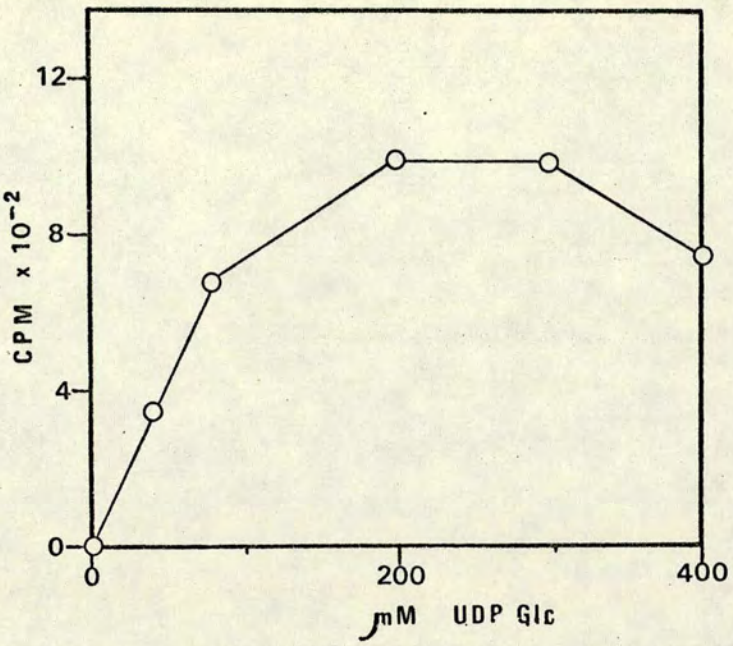


Fig. 44 Effect of substrate concentration on [^{14}C] incorporation from UDP[^{14}C]glucose into polymer by T646 membranes.

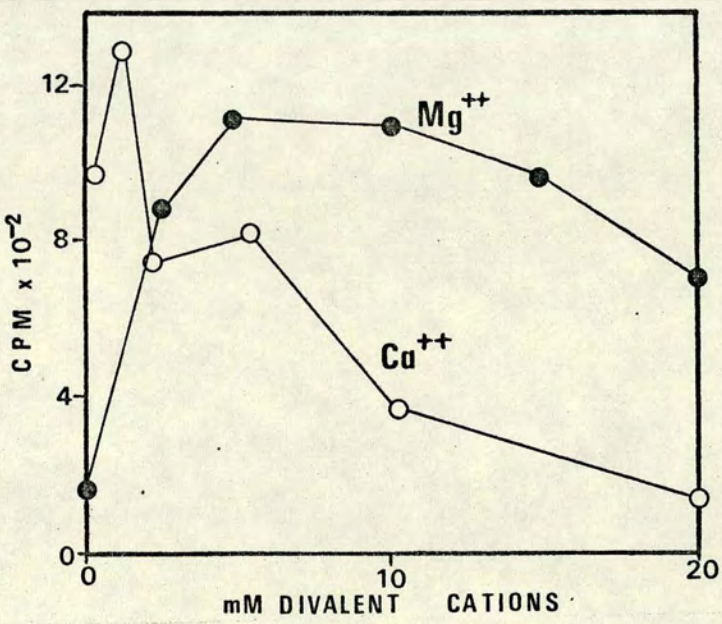


Fig. 45 Effect of divalent cations on [^{14}C] incorporation from UDP[^{14}C]glucose into polymer by T646 membranes.

The role of UDP-glucose as glucosyl donor was confirmed. Addition of unlabelled CDP-glucose or TDP-glucose had no effect on incorporation from UDP-[^{14}C]-glucose, while ADP-glucose slightly stimulated incorporation. Addition of similar levels of unlabelled UDP-glucose resulted in a 15% decrease, probably due to isotopic dilution. [^{14}C]-D-glucose could not replace UDP-[^{14}C]-glucose. It thus appeared that UDP-glucose was indeed the precursor of polymeric material.

b) Optimisation of Conditions for Polymer Synthesis by Particulate Preparations.

Low levels of radioactivity were routinely incorporated, into polymer therefore conditions were optimised with regard to polymer synthesis.

(i) Effect of Culture Age (fig. 40) : optimal incorporation of radioactivity (defined as cpm incorporated into polymer over 1h incubation), occurred with membranes prepared from early stationary phase cells. Initial high levels probably reflected the high concentration of older cells from the inoculum.

(ii) Effect of pH (fig. 41) : a broad pH optimum of 7.5-9.0 was observed, levels of incorporation were reduced by 50% below pH 7.5

(iii) Effect of Temperature (fig. 41) : routine incubations were carried out at 15°C. Radioactivity was incorporated over a wide temperature range of 5°-37°, but was maximum at 20°-30°C.

(iv) Effect of Enzyme Concentration (fig. 43) : with constant substrate and $MgCl_2$ concentrations, the incorporation of radioactivity into polymer increased linearly with protein or 'enzyme' concentrations 5-15 mg/ml.

(v) Effect of Substrate Concentration (fig. 44) : incorporation increased with substrate concentrations up to 150μM, but decreased above 300μM.

(vi) Effect of Divalent Cations (fig. 45) : incorporation of radioactivity into polymer was dependent upon Mg^{++}. Concentrations above 5mM Mg^{++} had little further stimulatory effect. Addition of Ca^{++} (calcium acetate) in the presence of 5mM Mg^{++} stimulated incorporation at low concentrations (up to 1mM) but was inhibitory at higher levels.

Addition of 10mmol EDTA resulted in the inhibition of enzyme activity.

(vii) Effect of Detergents : inhibition resulted from the addition of sodium sarcosinate or Triton X-100 even in trace quantities. Span-20 had little effect in low concentrations but was inhibitory at concentrations above 0.05%.

Subsequent incubation mixtures therefore contained 5mM $MgCl_2$, 2.5mM dithiothreitol, 200μM UDP-glucose, 0.5μCi/ml UDP-[^{14}C]-glucose and 10mg/ml protein (prepared from cells harvested in early stationary phase).

c) The Nature of Intermediates in in vitro Polymer Synthesis.

The involvement of CM soluble lipid intermediates has been demonstrated in washed cell exopolysaccharide synthesis and the involvement of similar compounds was demonstrated in in vitro systems.

(i) Effect of Bacitracin : Bacitracin caused inhibition of polymer and CM synthesis in washed cells ; similarly, in vitro polymer synthesis was sensitive to bacitracin. Incorporation of radioactivity decreased with increasing bacitracin concentration (fig. 46). Table 25 shows the effect of bacitracin on the incorporation of radioactivity into polymer by membranes from various Xanthomonas strains. All the strains were susceptible to the effect of bacitracin, but to variable degrees. The typical inhibitory effect of bacitracin (5mg/ml) on incorporation of radioactivity into polymer and CM material is illustrated in fig. 47.

(ii) Effect of Exogenous C_{55}-isoprenyl Phosphate Addition : Exogenous commercial C_{55}-isoprenyl phosphate was dissolved in CCl_4, added to incubation tubes and evaporated to dryness prior to the addition of other components. Addition of 10 n mol lipid phosphate had no detectable effect, but additions of 50 n mol and above stimulated the incorporation of radioactivity into CM extracts. Polymer synthesis was stimulated to a lesser degree than incorporation into CM (Table 26). The stimulatory effects were reversed by the addition of bacitracin.

(iii) Effect of Tunicamycin : The antibiotic tunicamycin acts at the lipid intermediate level in bacteria, specifically preventing the

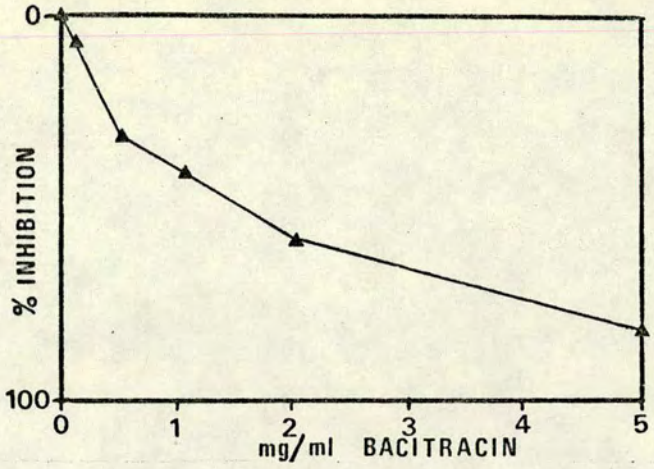


Fig. 46 Effect of bacitracin on [^{14}C] incorporation from UDP[^{14}C]glucose into polymer by 646D membranes.

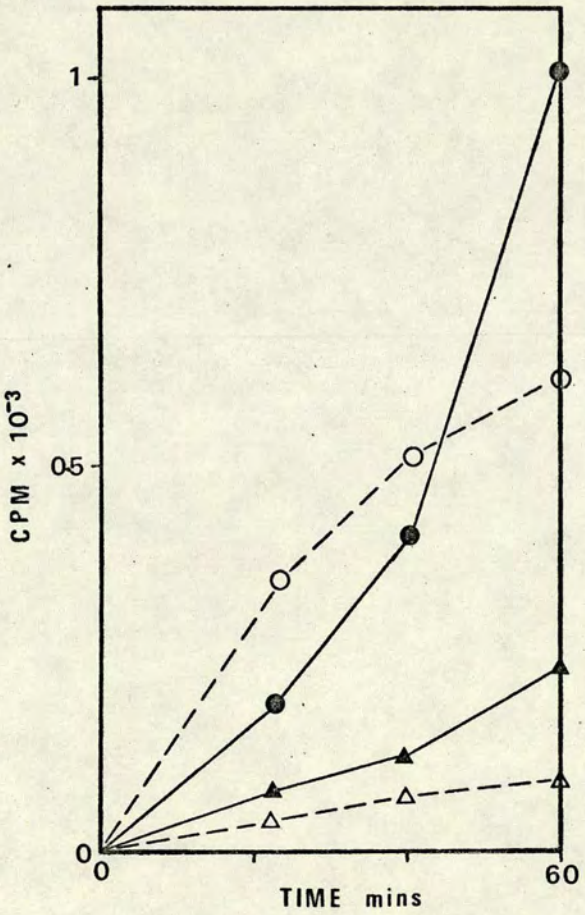


Fig. 47 Incorporation into polymer (●,○) and CM (▲, △) in the presence (--) and absence (—) of 1.0 mg/ml bacitracin by 646D membranes.

TABLE 25. Effect of Bacitracin on the Incorporation of Radioactivity from UDP-[^{14}C]-glucose into Polymer

STRAIN	% INHIBITION	
	1mg/ml bacitracin	5mg/ml bacitracin
T646	41.6	53.5
646D	39.6	84.0
646E	20.9	62.3
646KR	14.7	88.8

TABLE 26. Effect of Exogenous C_{55}-isoprenyl Phosphate on Radioactive Incorporation from UDP-[^{14}C]-glucose into CM and Polymer by T646 Membranes.

C_{55}-P CONCENTRATION (n mol)	% STIMULATION	
	Polymer	CM
10	0	0
50	74.8	19.1
100	90.8	229.6
500	238.2	759.9

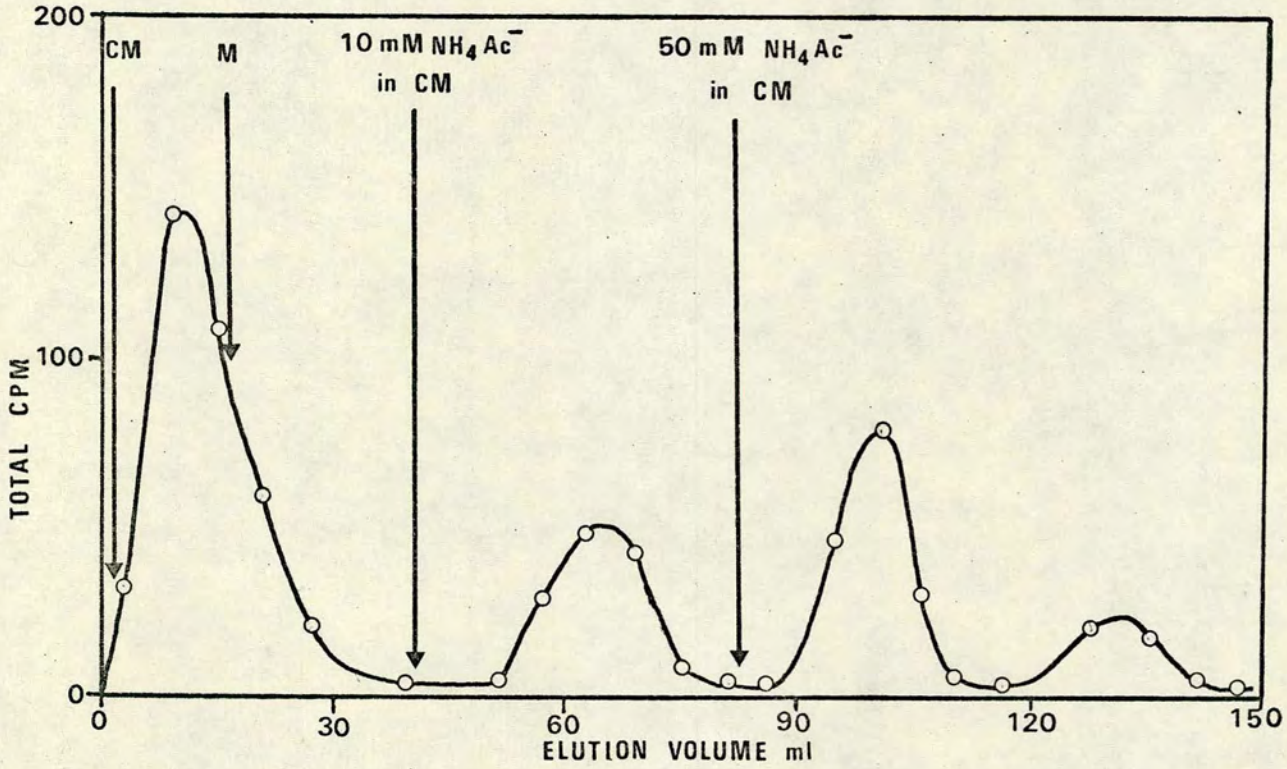


Fig. 48 DEAE cellulose elution profile of T646 in vitro CM extracts

transfer of N-acetyl glucosamine from its UDP precursor to lipid (Takatsuki and Tamura, 1971). The effect of this antibiotic on in vitro polymer synthesis was tested, since any residual cell-wall synthesising activity would be inhibited, possibly releasing more carrier lipid for exopolysaccharide synthesis. Tunicamycin concentrations of 10-500µg/ml had little effect upon the incorporation of radioactivity into either polymer or CM extracts however.

(iv) DEAE-cellulose Chromatography of in vitro Lipids : The incorporation of radioactivity into CM extracts was low in routine incubations. Pooled material was subjected to chromatography on DEAE-cellulose as described in the previous chapter. A typical elution profile is shown in fig. 48 ; similar results were obtained from each strain. Four peaks of radioactivity were eluted at similar elution volumes and ammonium concentrations to neutral lipid (A), lipid monophosphate galactose (B) and glucosyl-(C) and cellobiosyl-(D) lipid diphosphate derivatives.

Levels of radioactivity were such that each fraction was dried before radioactive counting, rather than counting a small aliquot. Thus no detailed characterisation was possible.

d) Reaction Sequences Involved in in vitro Polymer Synthesis. In an attempt to elucidate the sequence of reactions involved in the synthesis of in vitro polymer, the effect of sugars, sugar phosphates and nucleotides on polymer and intermediate synthesis was investigated.

(i) Effect of Uridine-5′-Monophosphate (UMP) : incorporation of radioactivity into polymer decreased with increasing UMP concentrations to a level of 60% of the control value in the presence of 5mM UMP (fig. 49). Polymer synthesis was inhibited to variable degrees in all the strains studied (Table 27). Addition of UMP to incubation mixtures, at a final concentration of 5mM, also inhibited the incorporation of radioactivity into CM by membranes prepared from each strain studied, a typical result is shown (fig. 50).

(ii) Effect of glucose-1-phosphate : glucose-1-phosphate inhibited the incorporation of radioactivity from UDP-[^{14}C]-glucose into polymer (fig. 51). All the strains studied were inhibited by final concentrations of 0.4 and 4.0mM glucose-1-phosphate, but to variable degrees

TABLE 27. Effect of UMP on Incorporation into Polymer.

	% INHIBITION	
STRAIN	0.4mM UMP	4.0mM UMP
T646	30.4	72.8
646D	9.0	34.8
646E	8.2	49.1
646KR	38.7	76.7

TABLE 28. Effect of Glucose-1-Phosphate on Incorporation into Polymer.

	% INHIBITION	
STRAIN	0.4mM glucose-1-phosphate	4.0mM glucose-1-phosphate
T646	16.6	21.0
646D	19.6	49.3
646E	38.6	68.3
646KR	15.0	61.7

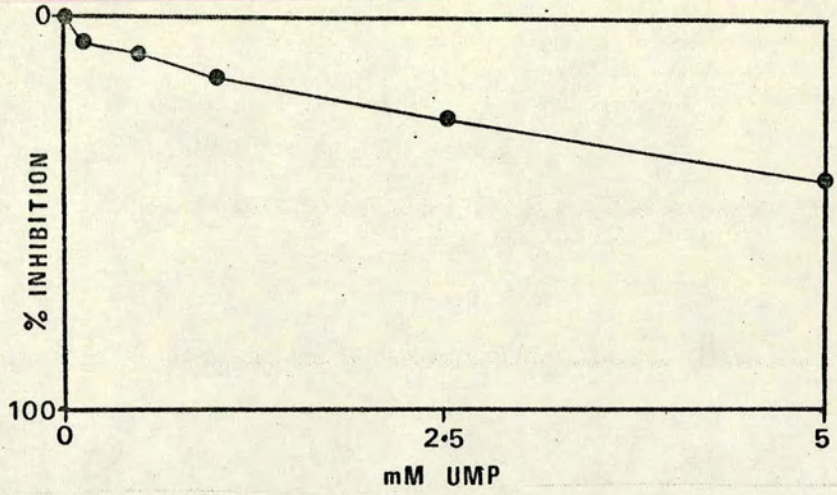


Fig. 49 Effect of uridine-5′-monophosphate on [^{14}C] incorporation from UDP[^{14}C]-glucose into polymer by 646D membranes

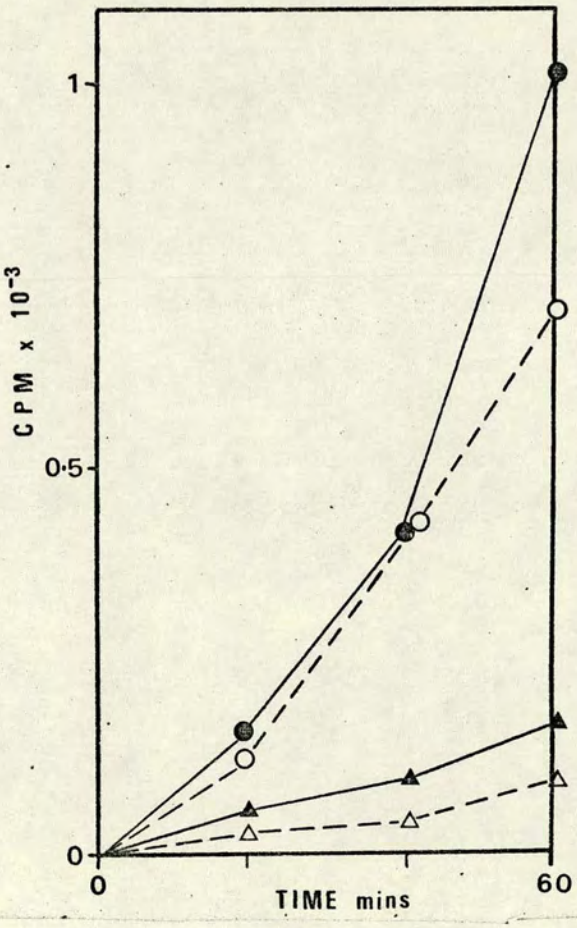


Fig. 50 Incorporation into polymer (●,○) and CM (▲,△) in the presence(- - -) and absence(——) of 4.0 mM UMP by 646D membranes.

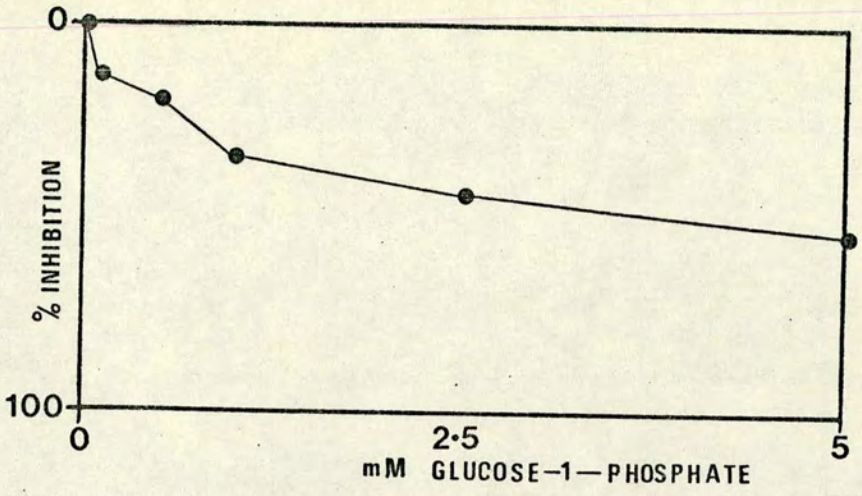


Fig. 51 Effect of glucose-1-phosphate on [^{14}C] incorporation from UDP[^{14}C]-glucose into polymer by 646D membranes.

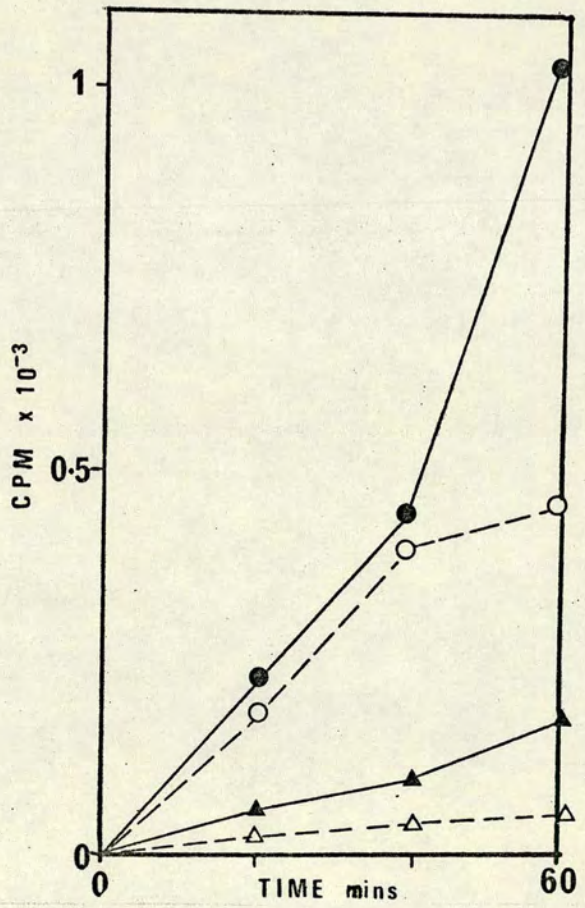


Fig. 52 Incorporation into polymer(●,○) and CM(▲,△) in the presence (- - -) and absence (——) of 4.0 mM glucose--1-phosphate by 646D membranes.

(Table 28). Membranes from the wild-type strain were less sensitive to 4.0mM glucose-1-phosphate, (20% inhibition) than those prepared from crenated strains (45%). Synthesis of CM material was also inhibited by glucose-1-phosphate, in each strain studied (fig. 52).

(iii) Effect of Uridine-5'-diphosphate (UDP) : addition of UDP to cell-free preparations from each strain resulted in a decrease in the incorporation of radioactivity into polymer (fig. 53). Membranes from T646 were inhibited by more than 90% in the presence of 0.4mM UDP (Table 29); preparations from the crenated mutants were less sensitive (10-35%) inhibition) under similar conditions. Addition of UDP also inhibited CM synthesis in each strain (fig. 54).

(iv) Effect of Glucose : incorporation into polymer decreased with increasing concentrations of glucose (fig. 55). The degrees of inhibition of incorporation into polymer, by each strain,are shown in Table 20. Addition of glucose inhibited incorporation of radioactivity into CM extracts (fig. 56) in all strains studied.

(v) Effect of Cellobiose : incorporation of radioactivity into polymer was stimulated by cellobiose addition,over the range 0-5mM (fig. 57); above 5mM, the response was not reproducible. Addition of 0.4mM cellobiose did not affect cell-free systems containing membranes from crenated strains but incorporation by T646 membranes was stimulated by 30%. At 4mM, incorporation by all the strains was stimulated (Table 31). Although polymer synthesis was stimulated by cellobiose, the incorporation of radioactivity into CM extracts from each strain was not affected (fig. 58).

(vi) Other additions : a variety of nucleotides including UTP, ATP, ADP, AMP, CDP and GDP had no effect on the incorporation of radioactivity into either polymer or CM extracts. Neither glucose-6-phosphate nor fructose-6-phosphate affected the incorporation of radioactivity into polymer in any of the strains studied.

e) The Nature of in vitro Products :

Following termination of incorporation by heating on a boiling water bath, incubation mixtures were sonicated to release any oligosaccharides from membrane material. Membranes were removed by

TABLE 29. Effect of UDP on Incorporation into Polymer.

STRAIN	% INHIBITION 0.4mM UDP	4.0mM UDP
T646	93.5	96.8
646D	13.7	48.0
646E	36.4	74.4
646KR	8.9	49.5

TABLE 30. Effect of D-glucose on Incorporation into Polymer.

STRAIN	% INHIBITION 0.4mM Glucose	4.0mM Glucose
T646	5.9	35.3
646D	25.8	51.6
646E	25.0	45.5
646KR	12.3	60.0

TABLE 31. Effect of Cellobiose on the Incorporation of Radioactivity from UDP-[^{14}C]-glucose into Polymer.

STRAIN	% STIMULATION 0.4mM Cellobiose	4.0mM Cellobiose
T646	30.1	64.2
646D	0	42.8
646E	0	45.7
646KR	0	16.3

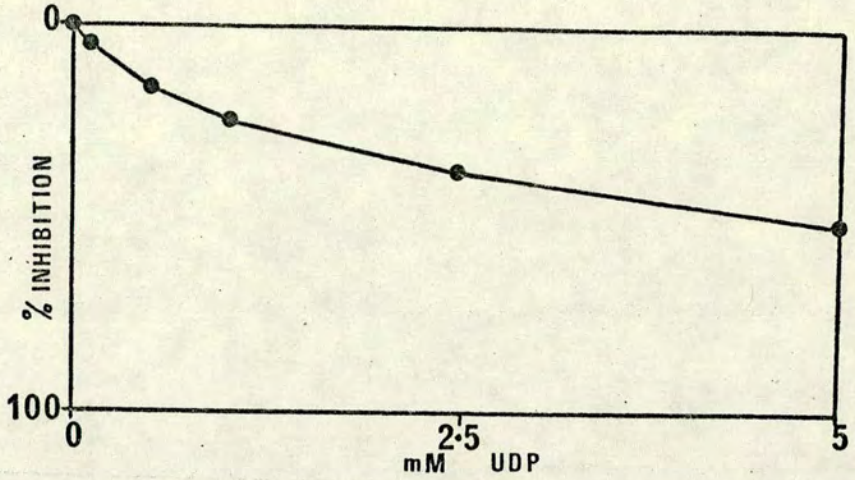


Fig. 53 Effect of uridine-5′-diphosphate on [^{14}C] incorporation from UDP[^{14}C]-glucose into polymer by 646D membranes.

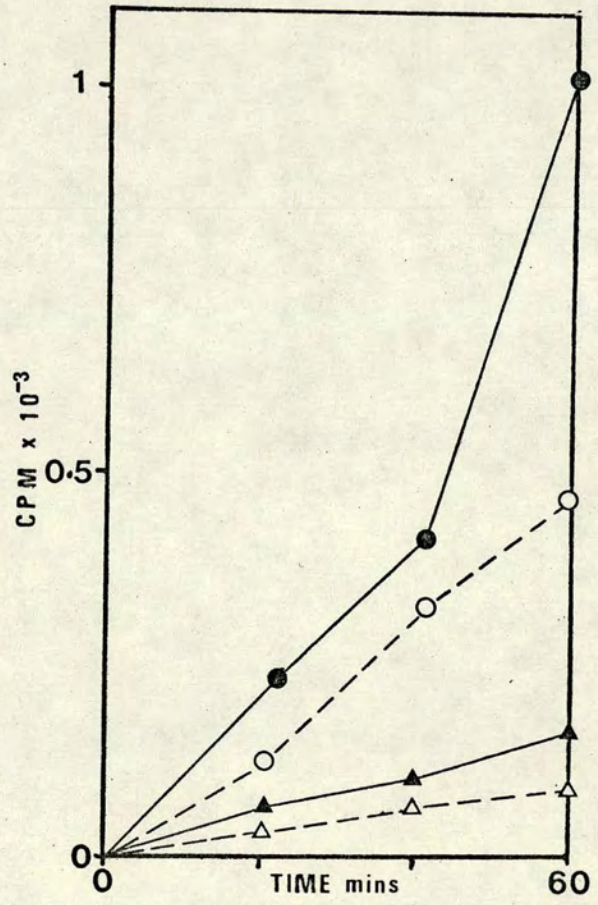


Fig. 54 Incorporation into polymer(●,○) and CM (▲,△) in the presence (- - -) and absence (——) of 4.0mM UDP by 646D membranes.

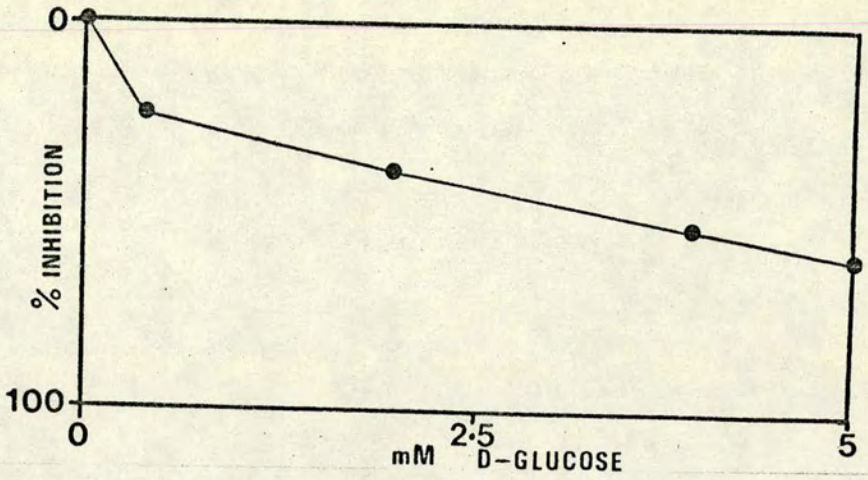


Fig. 55 Effect of D-glucose on [^{14}C] incorporation from UDP [^{14}C]-glucose into polymer by 646D membranes.

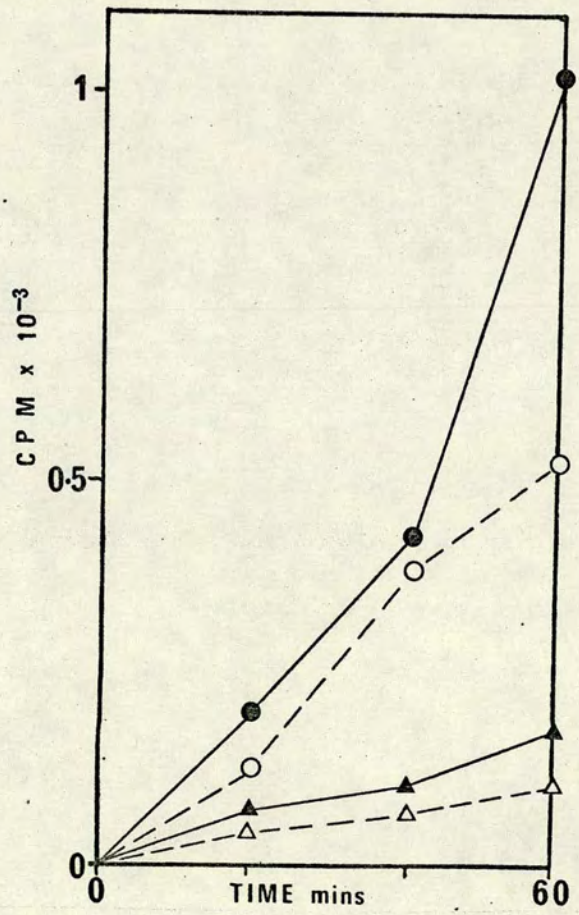


Fig. 56 Incorporation into polymer (●,○) and CM (▲,△) in the presence (---) and absence (——) of 4.0mM glucose by 646D membranes

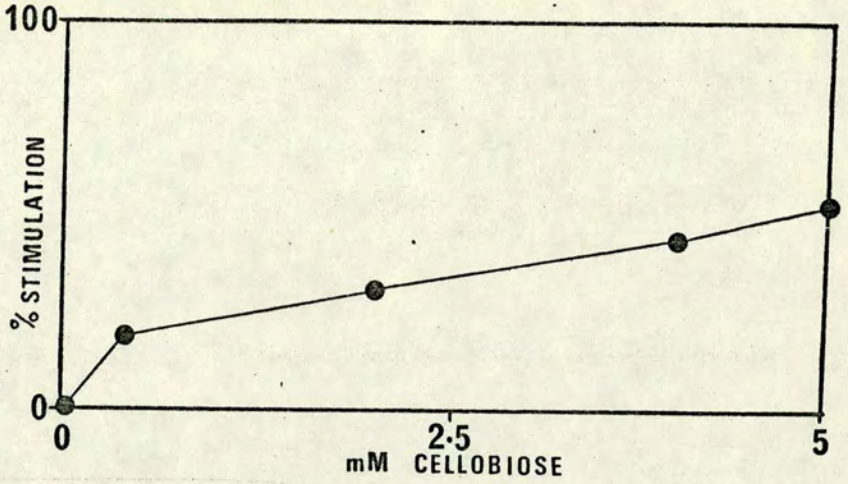


Fig. 57 Effect of cellobiose on [^{14}C] incorporation from UDP[^{14}C]-glucose into polymer by 646D membranes.

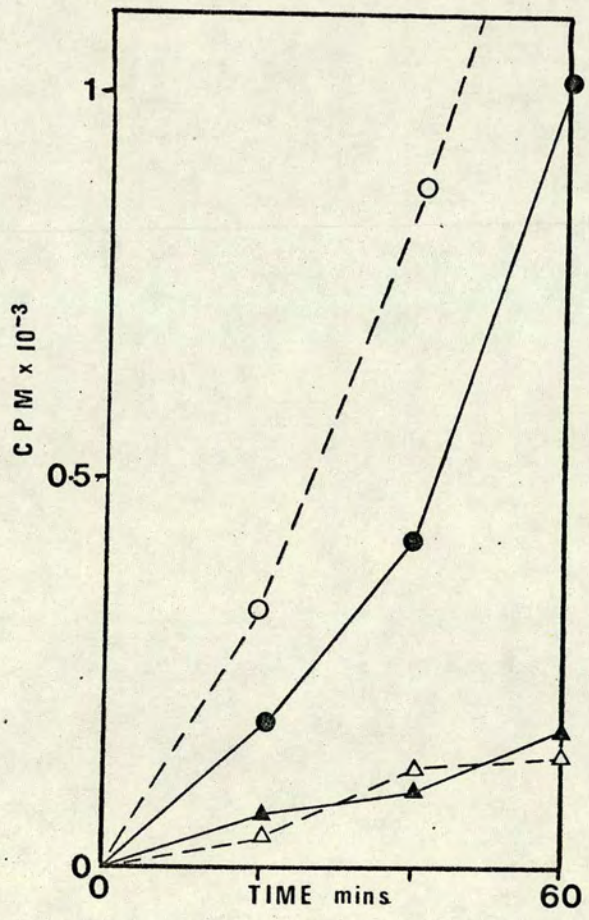


Fig. 58 Incorporation into polymer(●,○) and CM (▲,△) in the presence (– – –) and absence (——) of 4.0 mM cellobiose by 646D membranes.

centrifugation and the supernatants from parallel incubations pooled and reduced in volume. Use of large scale incubations reduced the yield of radioactive polymer.

Products from in vitro incubations were complex mixtures. After chromatography in solvent A, radioactive carbohydrate material with R_{glc} 1.0 , R_{glc} 0.69 (disaccharide) and R_{glc} 0.34 (trisaccharide) was detected; a wide band of radioactivity with R_{glc} 0-0.1 was also detected. The majority of these products were not assayed by chromatography in solvent D, since material smaller than tetra-saccharide was mobile in this solvent system.

In vitro products were separated by chromatography on Biogel P2 (30 x 1cm); distilled water was used as eluant at a flow rate of 10ml/h. Fractions of 500μl were collected and 50μl aliquots counted for radioactivity. Analysis of material from Xanthomonas T646 membranes (fig. 59) showed the presence of radioactive peaks co-chromatographing with standards of glucose, cellobiose and maltotriose. In addition material with molecular weight greater than UDP-glucose was detected. Peaks of radioactivity corresponded to carbohydrate material, detected by the phenol-sulphuric acid test.

A similar elution profile was obtained on chromatography of 646E in vitro products (fig. 59). Substantial amounts of radio-activity occurring in higher molecular weight material, might account for the apparently higher levels of radioactivity in 646E polymers assessed by chromatography in solvent D.

Hydrolysis of eluates from Biogel P2 (2N H_2SO_4 for 16h at 100°C) resulted in formation of a single compound with mobilities in solvents A and C, corresponding to glucose. Treatment with hexokinase produced a compound which remained at the origin in solvent A and had the electrophoretic mobility of hexose-phosphate. Under similar conditions, 0.1m mol of glucose was completely phosphorylated.

Treatment of in vitro products with commercial β-glucosidase or a partially purified commercial cellulase resulted in a quantitative increase in radioactive mono-, di- and, to a lesser extent, tri--saccharides, visualised by chromatography in solvent A. Little high molecular weight material (R_{glc} 0-0.1) remained after treatment with cellulase.

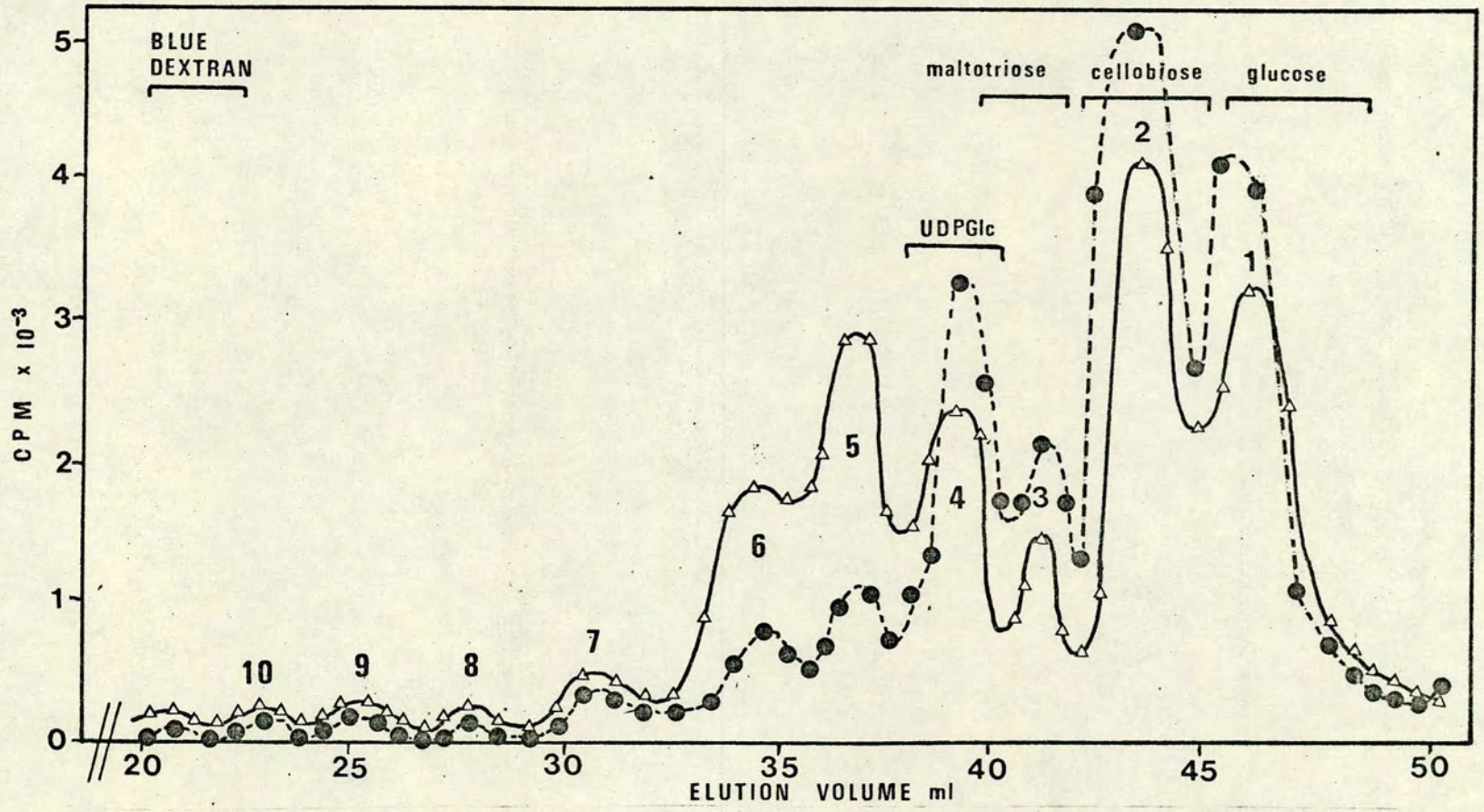


Fig. 59 Biogel P2 elution profile of <u>in vitro</u> products T646 membrane products 646E membrane products. For details see text.

The products were therefore identified as cellodextrins, oligosaccharides of variable chain length containing glucose as the sole monomeric constituent and probably linked in a β-configuration. In vitro products from membranes of each strain were of similar composition.

f) Attempts to Stimulate in vitro Polymer Synthesis by Addition of Acceptors.

The synthesis of a β-glucosyl polymer by membranes prepared from Xanthomonas strains suggested a similarity with Acetobacter xylinum. A. xylinum produces a pellicle of insoluble cellulose in vivo and in vitro preparations catalyse the formation of cello--dextrins. Cooper et al., (1975b) showed that addition of preformed cellodextrins to in vitro A. xylinum systems stimulated glucosyl transfer.

Membranes prepared from A. xylinum 1375 catalysed the incorporation of radioactivity from UDP-[^{14}C]-glucose into polymer and CM extracts at higher levels than Xanthomonas membranes, under similar conditions. Products were predominantly higher molecular weight (> disaccharide) oligosaccharides, on chromatography in solvent A. A parallel incubation containing unlabelled substrates was used to prepare cellodextrins.

Addition of cellodextrins from A. xylinum 1375 or Xanthomonas T646 and 646E in vitro incubations, to incubation mixtures containing T646 membranes, stimulated the incorporation of label from UDP-[^{14}C]--glucose into polymer. Preincubation with cold UDP-glucose ie prior synthesis of cellodextrins, was also stimulatory. Addition of in vivo polymers or cellulase degraded bacterial cellulose had no effect (Table 32A).

It was considered possible that prior synthesis of a β-glucose polymer was required before addition of 'xanthan' side-chains could occur. Thus T646 membranes were incubated with GDP[^{14}C]-mannose, UDP-glucose, UDP-glucuronic acid, phosphoenolpyruvate and acetyl CoA, with 'cellodextrin' additions. Cellodextrins from 646E and A. xylinum 1375, but not T646, in vitro incubations stimulated radioactive incorporation from GDP[^{14}C]-mannose into polymer (Table 32B); other additions had no effect. The levels of radioactivity were extremely

TABLE 32. Stimulation of Glucosyl Transfer (A) and Mannosyl Transfer (B) in Xanthomonas T 646 in vitro Systems, by 'Cellodextrins'.

ADDITION	A % STIMULATION OF INCORPORATION FROM UDP-[^{14}C]-GLUCOSE INTO POLYMER	B cpm (BACKGROUND CORRECTED) IN 20µl POLYMER SAMPLES AFTER 60 MIN INCUBATION
Control	0	0
T646 *in vivo* polymer	0	0
646E *in vivo* polymer	0	0
A. xylinum 1375 *in vivo* polymer	0	0
Cellulase hydrolysed bacterial cellulose	0	0
T646 *in vitro* products	23.9	0
646E *in vitro* products	53.0	11
A. xylinum 1375 *in vitro* products	44.1	15
Preincubation with 'cold' UDP-glucose	19.9	0

low, but reproducible and therefore significant. Reduction of 'enzyme' concentration decreased incorporation, but no increase in incorporation occurred if the enzyme concentration was increased. Due to low levels of incorporation, analysis of the product was not possible. Membranes from 646E did not catalyse mannosyl incorporation.

Attempts to demonstrate 'soluble' mannosyl-transferases failed. Addition of centrifugation supernatants did not facilitate transfer of label from GDP-[^{14}C]-mannose into polymer ; glucosyl transfer was reduced by addition of centrifugation supernatants.

No mannose containing nucleotide other than GDP-mannose was identified in Xanthomonas sp. nucleotide extracts (Results Section 3) Addition of UDP-mannose or CDP-mannose did not stimulate glucosyl transfer in T646 in vitro systems.

g) In vitro Polymer Synthesis by Toluene-Treated Cells.

Toluene-treated cells have been used in studies on nucleic acid synthesis in bacteria (Moses, 1974). Treatment with toluene permeabilises the cell to larger molecules but in vitro systems still maintain the vectorial properties of the cell. Toluene-treated Xanthomonas T646 cells were capable of UDP-glucose uptake whereas untreated cells were impermeable to this substrate.

Toluene-treated T646 cells synthesised radioactive polymer (chromatographically immobile in solvent D) from UDP-[^{14}C]-glucose. Addition of GDP-mannose, UDP-glucuronic acid, phosphoenolpyruvate acetyl CoA or cellodextrins did not affect glucosyl transfer.

Only small amounts of labelled product were obtained. Hydrolysis (2N H_2SO_4 for 16h at 100°C) led to the formation of [^{14}C]-glucose, as identified by chromatography in solvents A and C.

As a possible aid to de novo polymer synthesis, toluene-treated cells were stripped of any pre-existing xanthan, using depolymerase. After 2h exposure to the enzyme, cells were washed and incubated with UDP[^{14}C]-glucose, GDP-mannose, UDP-glucuronic acid, phosphoenol--pyruvate and acetyl CoA. The levels of radioactive incorporation were not improved however.

DISCUSSION

SECTION 1. Growth and Intermediary Metabolism of Xanthomonas Strains.

Xanthomonas campestris (Pammel) Dowson (Buchanan et al., 1974) is a gram-negative plant pathogenic bacterium causing vascular and parenchymatous diseases of brassicas. Plugging of vessels occurs due to the accumulation of acid mucopolysaccharide, pectins, melanins and wound gums (Sutton and Williams, 1969); these symptoms provide an important factor in the development of rot lesions. The production of exopolysaccharide by X. phaseoli has been correlated with pathogenesis (Corey and Starr, 1957).

Growth of X. campestris T646 in liquid media results in the production of highly viscous cultures; characteristic large mucoid colonies were evident on solid media. These observations were the result of production of copious amounts of exopolysaccharides. Release of slime polysaccharide into the medium occurred during exponential growth of X. campestris. Synthesis, or rather the capability to synthesise exopolymer was maximum during late exponential/early stationary phase and production of polymer ceased after 40-50h growth in nitrogen-limited batch cultures.

Exopolysaccharide production by crenated strains of X. campestris occurred at lower levels than the parent strain and under similar conditions, no detectable levels of polysaccharide were produced by Xanthomonas 646NM2. Growth of the crenated mutants occurred at a higher rate when compared with the wild-type strain and during growth, large decreases in culture pH were detected.

The maximum growth temperature for X. campestris T646 and 646NM2 was 30-32°C, however crenated mutants were able to grow, but produced no polymer, at 37°C. Many strains of X. campestris have been reported as capable of growth at 35°C (Buchanan et al., 1974).

Various nutritional studies involving X. campestris have concerned either the cultural conditions favourable for exopolymer production, in either batch or continuous culture (Moraine et al., 1966; Silman et al ., 1970; 1972), or the ability to produce xanthan from defined carbon sources (Souw and Demain, 1979). In contrast, the utilisation of supplied carbon sources in the production of energy for growth ie intermediary metabolism, has received relatively little

attention in X. campestris.

In early studies, Katznelson (1955; 1957) reported on the metabolism of gluconate by Xanthomonas sp.; although sonic lysates of X. campestris, X. pruni but not X. phaseoli were active, whole cells were not capable of gluconate metabolism. In a later study using radiorespirometric techniques, Zagallo and Wang (1967) showed that most glucose (80-90%) was catabolised via the Entner-Doudoroff pathway with the Pentose Phosphate pathway fulfilling a minor role in several Xanthomonas sp. studied. These observations suggested a similarity between X. campestris and the system more recently proposed for glucose metabolism in Ps. aeruginosa [Ng et al.,(1973) and see fig. 21]. Since X. campestris is classified within the Pseudomonadaceae (Buchananan et al.,1974) with a G+C value of 63.5 - 69.2% over 29 nomen species, compared to 67% in Ps. aeruginosa, the similarities are perhaps not surprising.

Analysis of the amounts of glucose metabolising enzymes demonstrated differences between the Xanthomonas strains studied here. Crenated mutants appear to have higher amounts of the enzymes involved in the oxidation of glucose to gluconate and subsequently to 2-oxogluconate. Operation of this essentially periplasmic system might account for the significant fall in the pH of the culture medium during growth; amounts of gluconate have been isolated from the growth medium. Wild-type T646 and the non-mucoid mutants were able to grow on gluconate but, unlike the crenated mutants, only poorly on 2-oxogluconate. The apparent lack of glucose dehydrogenase activity in T646 and the non-mucoid strains would rule out the periplasmic oxidative pathway in these strains. Growth of T646 on gluconate results in detectable activity of gluconokinase and gluconate dehydrogenase and increased amounts of enzymes involved in metabolism of 2-oxogluconate. This would indicate operation of the periplasmic pathway after the glucose dehydrogenase 'block'.

All the strains studied possess enzymes of the intracellular phosphorylative pathway. Therefore Xanthomonas strains resemble Ps. aeruginosa in possession of two coupled, yet discrete systems for glucose transport. The intracellular pathway in Ps. aeruginosa exhibits a low km for glucose but with a broad specificity ; the

extracellular pathway has a high km for glucose with a requirement for glucose dehydrogenase activity (Midgley and Dawes, 1973). In the presence of high concentrations of glucose, periplasmic gluconate production inhibits the intracellular pathway. Since T646 and 646NM2 possess no detectable glucose dehydrogenase activity, such inhibition would not occur during growth on glucose, the substrate being transported and directly phosphorylated. Under carbon limited conditions, Ps. aeruginosa transports glucose by the intracellular pathway (Whiting, Midgley and Dawes, 1976).

Growth of Ps. aeruginosa on organic acids (eg. citrate, succinate) results in repression of glucose metabolising enzymes (Hamilton and Dawes, 1960 ; Hamlin, Ng and Dawes, 1967). This occurs to a slight extent in X. campestris. Growth on glycerol led to derepression of glucose metabolising enzymes in Ps. aeruginosa (Hamlin et al., 1967).

The glycolytic pathway does not function in X. campestris due to lack of the phosphofructokinase step, a result in agreement with an early study involving X. phaseoli (Hochster and Katznelson, 1958). The 6-phosphogluconate produced by glucose metabolism is further metabolised to produce pyruvate in all the Xanthomonas strains studied. Only the crenated mutants possess detectable decarboxylating 6-phosphogluconate dehydrogenase activity indicative of active Pentose Phosphate Pathway.

Thus, the crenated mutants have a more complex system of glucose metabolism, than do either the parent or non-mucoid strains. Since little difference is noted in activities of enzymes of the tricarb--oxylic acid cycle, the only form of terminal oxidation in these bacteria, the effect of such differences in terms of cell growth, is difficult to interpret.

The ability of these strains to grow on glycerol tends to suggest a role for reversed glycolysis, in order to produce sugar phosphates for the synthesis of cellular constituents. Thus some mechanism must exist, whereby the glycolytic pathway can by-pass inactive phosphofructokinase in the reverse direction.

The active transport system for D-glucose in Ps. aeruginosa has a broad specificity since it is also capable of transporting

α-methyl-D-glucoside, 2-deoxy-D-glucose, D-xylose, D-glucosamine and D-galactose. No phosphoenolpyruvate-dependent phosphotransferase systems operate in Ps. aeruginosa (Phibbs et al., 1970 ; Romano et al., 1970 ; Midgley et al., 1973). Studies with membrane vesicles prepared from Xanthomonas T646 confirm that no phosphenolpyruvate-driven transport systems function in the uptake of several tested hexoses. This result may be very significant since the exopolysaccharide produced by T646 contains pyruvate.

If the precursor of polymeric ketal groups is, as postulated, phosphoenolpyruvate, then operation of phosphoenolpyruvate-dependent hexose uptake systems would diminish the intracellular pool of available phosphoenolpyruvate. Therefore the degree of polymer pyruvylation would be decreased. No evidence is available concerning the transport systems involved in hexose uptake in other bacteria which produce pyruvylated exopolysaccharides.

In retrospect, results concerning the absence of phosphoenol--pyruvate-dependent phosphotransferase systems in X. campestris are not too surprising. Metabolism of glucose via the Entner-Doudoroff pathway would produce 1 mol pyruvate per mol glucose, conversion by glycolysis would yield 2 mol pyruvate however. Thus the metabolic pathways of X. campestris impose their own restraint upon the levels of available phosphoenolpyruvate. Most studies have concerned enteric bacteria, it would be interesting to ascertain whether all bacteria possessing phosphoenolpyruvate-dependent phosphotransferase systems also possess glycolytic activity.

SECTION 2. The Cell Surface of Xanthomonas campestris.

X. campestris T646 produces large mucoid colonies when grown on YE plates. Mutagenic treatment of the wild-type strain resulted in the isolation of two classes of mutant which differ fundamentally in their appearance on YE plates.

a) those mutants in which the ability to synthesise exopoly--saccharide is lost, typified by the non-mucoid 646NM2.

b) those mutants which produce exopolysaccharide at lower levels than the parent strain and produce unusual crenated colonies ; typified by the crenated mutants 646D, 646E and 646KR.

a) Non-mucoid mutants

Analysis of the wild-type lipopolysaccharide from Xanthomonas T646 revealed glucose to be the predominant constituent (94.6%) with only trace amounts of galactose (3.9%) and rhamnose (1.6%). The lipopolysaccharide of X. campestris 646NM2 differed significantly from the wild-type, since rhamnose was the major component (61.3%) with galactose (20.0%) and glucose (9.8%) also present. Thus the composition of 646NM2 lipopolysaccharide resembles more closely the published X. campestris lipopolysaccharide compositions. Water soluble lipopolysaccharides from X. campestris have been reported to contain rhamnose, galactose, glucose, KDO ; certain Xanthomonas sp. (but not X. campestris) also contain either fucose or xylose (Volk 1968b ; Schlabach, 1970).

No data is available as to the proportions of sugars in water soluble lipopolysaccharides, but in a report concerning phenol--soluble material, Hickman and Ashwell (1968) reported rhamnose (58%) 3-acetamido-3,6-dideoxy-D-galactose (25%) ; glucose and galacturonic acid, but not KDO, were also present. Galacturonic acid-1-phosphate has been detected in water-soluble lipopoly--saccharides from X. campestris (Volk, 1968a). Configurations of rhamnose residues in aqueous and phenol soluble material were opposites (Volk, 1968b).

No evidence was found in this study for the presence of galacturonic acid-1-phosphate in water soluble lipopolysaccharide. It is however possible that such residues were lost during preparation, since the linkage has been reported to be labile, especially

in acid (pH 3.3) conditions (Volk, 1968b).

It is likely that the appearance of trace amounts of ribose in lipopolysaccharide hydrolyrates and the unusually high yield values,results from extensive contamination with nucleic acids.

Analysis of the membrane proteins of 646NM2 showed little difference from the wild-type band pattern. Small amounts of exopolysaccharide produced were, when analysed, chemically identical with the wild-type product.

b) Crenated Mutants.

Investigation of the antibiotic sensitivites of the crenated strains showed differences in the sensitivity to cell wall active antibiotics (Bacitracin,, D-cycloserine, Vancomycin, Penicillin and Novobiocin) when compared with the wild-type strain. These mutants showed also increased resistance to Polymyxin B, as did the non-mucoid 646NM2, suggesting alterations in the cell surface.

Polymyxin B is a surfactant capable of disrupting surface structures, particularly the lipopolysaccharide molecule (Cooperstock, 1974 ; Wade, Brown and Tsang, 1975) and thus affecting osmotic stability of the cell. Schlecht and his co-workers (Schlecht and Schmidt, 1969 ; 1970 ; 1972 ; Schlecht and Westphal, 1970) have described the antibiotic and dye sensitivities of Salmonella sp. lipopolysaccharide mutants and several independent studies have revealed similar effects. "Deep-rough" mutants deficient in heptose (Salmonella Re mutants) were found to be more sensitive to bacitracin, novobiocin, polymyxin, vancomycin and certain penicillins (Roantree, Kuo and MacPhee, 1977). Previous reports (Tamaki, Sato and Matsuhashi, 1971) suggested that increased sensitivity was due to a lack of phosphate diester linkages capable of forming a network of surface lipopolysaccharide.

Little is known of the composition of Xanthomonas core polysaccharides, however most workers suggest that heptose is absent, indicating an obvious difference from enteric bacteria. In contradiction however Volk (1966) has described a X. campestris strain containing both heptose and phosphate. No heptose was detected in the strains in this study. The inner core region of Xanthomonas strains appears to contain a single KDO residue substituted with

mannose-1-phosphate (Volk et al., 1972). Hickman and his co-workers (Hickman et al., 1966) reported the absence of phosphate in Xanthomonas sp., thus it is unlikely that the phosphate diester linkages reported in enteric bacteria would exist in these strains.

The lipid-A fraction of a strain of X. campestris (X. sinensis) has been reported to be similar to that of Salmonella minnesota, E. coli and Rhodopseudomonas gelatinosa (Hase and Rietschel, 1976). All these strains possessed a β1',6-linked diglucosamine unit and two phsphate residues, one glycosidically linked and the second, ester-linked to the non-reducing glucosamine residue. Cross-linking of diglucosamine units by 1,4' pyrophosphate bonds has been reported in certain enteric bacteria (Rothfield et al., 1971).

Despite the data concerning the composition of O-antigen, and to a lesser extent, core and lipid-A, little if anything, is known about the precise structure of Xanthomonas sp. lipopolysaccharides.

Composition of the O-antigen chains in crenated mutants is similar to those observed in 646NM2, thus differing from the parent strain. Chromatography of partially hydrolysed lipopolysaccharide from crenated strains, indicated the absence of extensive side chain substitution but the observation of 'soluble' polysaccharides chemically identical with O-antigen suggests that the side-chains are polymerised. Thus attachment of O-antigen to core is probably defective in crenated strains, analogous to either Salmonella Rfa mutants in which the core is defective, or alternatively, the RfL mutants in which O-antigen transferases are defective (Stocker and Mäkelä, 1978).

Analysis of membrane protein patterns showed that in addition to changes in concentrations of specific proteins (determined on the basis of response to Coomassie blue staining), gross changes were also detected. It is possible that these changes are related to changes in the lipopolysaccharide fraction. Loss of outer membrane proteins has been shown to accompany lipopolysaccharide changes in S. typhimurium and E. coli (Ames et al., 1974 ; Chatterjee, Ross and Sanderson, 1976; Koplow et al., 1974). These changes were correlated with the release of periplasmic enzymes into the growth medium. Although there is no evidence for this phenomenon in the crenated mutants of X. campestris.

the appearance in the growth medium, of gluconate produced periplasmically during growth on glucose, might be an indication of 'leakiness' in these strains. Whether such changes result in the cell being more permeable to potential substrates, has not been established.

The mode in which proteins, phospholipids and lipopolysaccharides interact in the outer membrane of bacteria is not clear. Similarly, the manner in which the phenotypic changes reported in Xanthomonas crenated strains influence antibiotic sensitivity are difficult to reconcile, particularly since the changes reflect both increased resistance and increased susceptibility to specific antibiotics.

The report of an Re mutant of S. typhimurium which exhibits altered colonial morphology (Wilkinson, Gemski and Stocker, 1972) is perhaps significant with regardto the Xanthomonas crenated strains. The ability of crenated strains to grow at 37°C may also be related to changes in the cell surface. Bacteria unable to grow at elevated temperatures have been reported, possibly due to the production of thermosensitive components. Changes involving an elevation in growth temperature are more difficult to explain, however. One possibility could be that a mutation in the crenated mutants affects a gene regulating production of a gene product which is not required for growth at 30°C, but is an absolute requirement for growth at 37°C. Certain lipopolysaccharide mutants of S. typhimurium show a temperature sensitive response, a lipopolysaccharide containing heptose is required for growth at 42°C but is not essential for growth at 30° or 37°C (Chatterjee, Sanderson, Ross, Schlecht and Lüderitz, 1976). The nature of the required product in Xanthomonas sp. has not been identified though it would appear that in analogy with Salmonella sp., the structure of the lipopolysaccharide core is important.

Chemical analysis of the exopolysaccharides from the crenated strains suggested a composition different from the wild-type product and furthermore, slight variations between the composition of different batches from the same strain.

High levels of lipopolysaccharide specific sugars were detected in the exopolysaccharide, but the origin of these sugars remains

unclear. It is possible that changes in the cell surface led to the release of lipopolysaccharide-enzyme complexes (Rothman et al., 1969 ; Knox et al., 1967) during growth. There is however no evidence to support this suggestion in Xanthomonas sp. It is also possible that formaldehyde treatment, during harvesting of cultures from enamel trays, was inadequate, resulting in cell-surface damage and release of surface material at the vigorous homogenisation step.

Galactose has previously been reported in the exopolysaccharide of X. stewartii (Gorin and Spencer, 1961) and Xanthomonas strain S19 (Fareed and Percival, 1976). Studies with X. fuscans, a sub-species of X. campestris (Konicék, Lasick and Wurst, 1977) have indicated the presence of α-and β-glucose, α-and β-mannose, with small amounts of 6-deoxy-L-mannose and D-glucuronic acid in exopolysaccharide. Ribose, possibly from nucleic acid contamination, was also detected.

Recently, a strange exopolysaccharide closer in its composition to lipopolysaccharide was identified in a Xanthomonas sp. isolated from soil (Yadomae, Yamada, Miyazaki, Omori and Hirota, 1978). The polysaccharide contained glucose, mannose, O-acetyl manno-octulosonic acid and an unidentified deoxyhexose. No glucuronic acid or pyruvate were detected and no lipid or phosphate were present. The polysaccharide was unstable in 2% acetic acid at 100°C. No evidence is available concerning the cell wall lipopolysaccharides of this strain for comparative purposes.

Williams et al., (1977 ; 1978) have reported on the factors influencing slime exopolysaccharide production by Pseudomonas PB1 (NC1B 11264) in batch and continuous culture. The chemical composition and rheological properties of the polysaccharide have recently been studied (Williams, Wimpenny and Lawson, 1979). Chemically, the exopolysaccharide is a galactoglucan containing acetate and pyruvate, but contamination by rhamnose and mannose occurred. The presence of rhamnose was ascribed to either cellular contamination (Eagon, 1956) or release of a rhamnosyl-glycolipid, identified in these strains (Brown, Foster and Clamp, 1969 ; Drewry, Symes, Gray and Wilkinson, 1975). Mannose and rhamnose contamination was reduced after precipi-

-tation with cetyltrimethylammonium bromide. Although rhamnosyl--glycolipids have not been reported in X. campestris, the described similarity between the genera Xanthomonas and Pseudomonas suggests that the presence of rhamnosyl-glucolipids in X. campestris should not be ruled out.

Analysis of crenated exopolysaccharides with a crude but specific depolymerase indicated the presence of some exopolysaccharide material resembling the wild-type products. Changes in ratios of major hydrolytic fragments were related to alterations in acylation. No acetate and only trace quantities of pyruvate were detected chemically. These results exemplify the advantage of specific polysaccharases as initial screening tools in exopolysaccharide analysis.

The observation of higher levels of glucose than mannose in the hydrolytic fragment F, suggests a further difference in the xanthan-fraction of the crenated exopolysaccharides. Research continues into this aspect. Treatment of crenated exopolysaccharides with cellulase, releases small amounts of glucose from the polymer. (I W Sutherland, personal communication). The increased glucose content might suggest a shortage of side chains, resulting in lower amounts of mannose. This would account for the reduced acetate, pyruvate and glucuronic acid content. Alternatively, these strains may produce two poly--saccharides, one substituted and the second unsubstituted. Current studies are hampered by the lack of (i) a highly active cellulase preparation and (ii) an enzyme capable of releasing every second glucose residue on the polymer 'backbone', which would allow analysis of the released substituted on non-substituted disaccharide.

Towards the end of this study, a second xanthan depolymerase was obtained from a laboratory isolate. The enzyme was intracellular unlike the extracellular enzyme from isolate H. The second isolate was however a mixture of at least two distinct organisms. Removal of any component organism resulted in loss of depolymerase activity. Preliminary experiments indicated that the hydrolytic products of the depolymerase activity were qualitatively similar from each source.

Alterations in ketalation of X. campestris exopolysaccharide have been reported previously (Sandford et al., 1976 ; Davidson, 1978).

Growth of X. campestris T646 in media supplemented with different carbon sources also led to a variation in pyruvate content. These results together with data obtained with crenated mutants, might be significant with respect to the use of xanthan gum as a food additive. Guidelines are laid down for the use of polysaccharides as food additives; the Food and Agriculture Organisation of the United Nations World Health Organisation (F.A.O., 1975) state that xanthan gum should contain not less than 1.5% pyruvate.

Although molecular weights for xanthan have appeared in the literature (see Introduction, Table 3), no data concerning molecular weight has been obtained for the strains studied here. Recently, Hisamatsu, Abe, Amemura and Harada (1978) have described the synthesis of an oligosaccharide, the repeating unit of succinoglucan by a mutant of Alcaligenes faecalis var myxogenes. The wild-type organism produced a similar product (degree of polymerisation 9) but reduced amounts of high molecular weight (degree of polymer--isation 1600) succinoglucan in the presence of penicillin or vancomycin. The authors suggest that the wild-type observation was due to the antibiotics causing a reduction in carrier lipid available for polymer synthesis, but did not take into account possible effects upon the cell surface resulting from growth in the presence of the antibiotic. It is possible that alterations in the cell surface of Xanthomonas crenated strains might lead to defects in polymerisation of exopolysaccharide, an effect which would not be recognised from hydrolysis and depolymerisation data.

Attempts to chromatograph Xanthomonas sp. exopolysaccharides failed in this study. Due to high molecular weight, products were excluded from Sephadex G200 and other commercial dextrans.

SECTION 3. Control of Exopolysaccharide Synthesis.

X. campestris produces large amounts of exopolysaccharide during exponential growth. Analysis of the lipopolysaccharide produced by these strains indicates the presence of common monomers and hence a requirement for common precursors.

In Xanthomonas T646, glucose-1-phosphate appears to occupy a central role. By the action of constitutive glucose pyrophos--phorylase, glucose-1-phosphate can be converted into UDP-glucose. Further activity of UDP-glucose dehydrogenase or UDP-galactose-4-epimerase would produce UDP-glucuronic acid and UDP-galactose from UDP-glucose substrate. Conversion of glucose-1-phosphate to dTDP-glucose and subsequently to dTDP-rhamnose requires a multi--enzyme system of epimerases and reductases and possibly involves a complex intermediate dTDP-4-keto-6-deoxy-D-glucose (Okazaki et al., 1962).

The production of a lipopolysaccharide containing 95% glucose in the polysaccharide moiety and an exopolysaccharide which contains 2 moles of glucose per repeating unit during growth, suggests control of the fate of glucose-1-phosphate. Conversion of glucose-1--phosphate to dTDP-rhamnose would produce a precursor which can only be involved in lipopolysaccharide. Regulatory control of this conversion would leave UDP-glucose available for either polysaccharide and would thus account for the 'replacement' of lipopolysaccharide-rhamnose with glucose and perhaps galactose.

Recently, a group of closely related oligosaccharides have been isolated from E. coli (Van Golde et al., 1973). These membrane derived oligosaccharides (M.D.O) are highly branched $\beta 1 \rightarrow 2$ and $\beta 1 \rightarrow 6$ linked oligosaccharides containing 8–10 glucose units with succinyl-O ester residues and containing sn-glycero-1-phosphate, derived from the polar head groups of phosphatidyl glycerol and/or cardiolipin and phosphatidylethanolamine. The oligosaccharide moiety is derived from UDP-glucose (Schulman and Kennedy, 1977). These molecules are thought to be located, either periplasmically to aid osmotic stability, or possibly, but less likely, associated with the outer membrane (Schulman and Kennedy, 1979). The authors have inferred a general occurrence in gram-negative bacteria.

There is however, no evidence for the presence of similar ethanol soluble matererial in X. campestris. This is perhaps not surprising given a heavy demand for cellular UDP-glucose. Whether other oligosaccharides e.g unattached O-antigen, replace the functional role of MDO in X. campestris is not known.

Analysis of biosynthetic enzyme levels in the non-mucoid strains did not indicate a defect capable of accounting for the non-mucoid phenotype. This result is in agreement with reports concerning Salmonella O-antigen biosynthesis, in which mutations affecting later stages of biosynthesis occur more frequently than do mutations affecting enzymes involved in precursor synthesis (Nikaido, 1968). It is likely that the lesion in these non-mucoid mutants affects a specific transferase or "polymerase". Synthesis of exopolysaccharide in X. campestris might be expected to require five transferases, two capable of transferring glucosyl residues, two for mannosyl residues and one for glucuronosyl residues. The nature of the "polymerase" in bacterial polysaccharides has yet to be resolved. How such mutations fit with the observation that 646NM2 produces small quantities of authentic exopolysaccharide, under conditions where no reversion to wild-type phenotype has been achieved, is not clear.

It is conceivable that in the non-mucoid mutants, where UDP-glucose, GDP-mannose and UDP-glucuronic acid are synthesised and enter the nucleotide pool, accumulation would occur in the absence of exopolysaccharide synthesis. Such accumulation is not however evident from the analysis of nucleotide pools in 646NM2, suggesting that some form of feedback inhibition may occur. Similar systems of control have been reported in TDP-glucose and UDP-glucose pyrophosphorylases in E. coli (Bernstein and Robbins, 1965) and in the synthesis of GDP-fucose (Kornfeld and Ginsburg, 1966) for incorporation into E. aerogenes exopolysaccharide.

Crenated mutants of X. campestris produce lower levels of exopolysaccharide than does the parent strain and a lipopolysaccharide which resembles the non-mucoid mutant but is apparently 'rough' or 'semi-rough'.

Markovitz and his colleagues (see Markovitz, 1977) have described

a regulatory system for colanic acid synthesis in E. coli. The amounts of enzymes involved in sugar nucleotide biosynthesis were regulated by products of genes coded on a plasmid. Mutations at three distinct loci designated cap R, cap S and cap T, resulted in derepression and expression of the mucoid phenotype. Thus in E. coli, control of exopolysaccharide synthesis is at the genetic level. An independent study has shown that a similar regulatory system operates in control of colanic acid synthesis by E. coli, S. typhimurium and A. cloacae (Grant, Sutherland and Wilkinson, 1970).

Although plasmids have been detected in X. campestris (R E Cripps, personal communication), their involvement in the regulation of exopolysaccharide biosynthesis has not been established. Elimination of a plasmid carrying genes regulating the synthesis of UDP-galactose or dTDP-rhamnose provides an attractive, if speculative, explanation for the altered lipopolysaccharide in the crenated strains and the non-mucoid mutant. In colanic acid synthesis (Grant et al., 1970), although UDP-glucose pyrophosphorylase was constitutive, derepression of UDP-galactose-4-epimerase was reported.

The apparent stability of the Xanthomonas mutants studied, despite repeated mutagenic treatments in an attempt to revert mutants to the wild-type phenotype, tend to support the plasmid theory. Furthermore the three crenated mutants were derived from the single parent strain using different mutagenic treatments; indeed 646D was the only spontaneous mutant isolated during this study. These strains were phenotypically similar and it would be unlikely that three independently isolated mutants would all be affected at the same chromosomal locus.

Control of exopolysaccharide synthesis at the metabolic level may also occur in Xanthomonas sp. During exponential growth of Xanthomonas T646, significant amounts of metabolic energy must be channelled into exopolysaccharide biosynthesis. It is perhaps not surprising that extra pathways of glucose metabolism, ie. periplasmic oxidation of glucose and the pentose phosphate pathway, do not operate in the mucoid wild-type organism.

Use of the direct phosphorylative pathway in X. campestris T646 results in most transported glucose forming glucose-6-phosphate.

The glucose-6-phosphate may be subsequently metabolised via 6-phosphogluconate and the Entner-Doudoroff pathway, to pyruvate. Alternatively, glucose-6-phosphate can be converted to glucose-1--phosphate and thus enter into sugar nucleotide biosynthetic sequences.

In the crenated mutants, the operation of the periplasmic pathway, possibly as a result of regulatory mutations would convert a certain amount of transported glucose either directly or indirectly, via 2-oxogluconate-6-phosphate, to 6-phosphogluconate. Thus glucose metabolism in this fashion would not involve glucose-6-phosphate formation and would not therefore become available to poly--saccharide biosynthesis. During growth, synthesis of cell wall and lipopolysaccharide would receive priority in order to maintain cellular viability. Thus synthesis of exopolysaccharide would be dependent upon the amounts of glucose-6-phosphate remaining.

The extra flow through catabolic pathways could be diverted through the Pentose Phosphate pathway. The net result in terms of the expected phenotype, would be an increased growth capability but reduced exopolysaccharide biosynthesis, as observed in the crenated mutants.

SECTION 4. Exopolysaccharide Biosynthesis in X. campestris.

Many independent studies have established the involvement of carrier lipids in the assembly of bacterial polysaccharides (see Introduction, Section 3). Most recently, lipid intermediates have been implicated in the synthesis of capsular exopolysaccharides in E. aerogenes (Sutherland et al., 1970 ; Troy et al., 1971.) and E. coli (Johnson et al., 1977). In each case, the lipid was identified as C_{55}-undecaprenol phosphate.

Radioactivity was incorporated into CM extracts from X. campestris washed cells, under conditions where radioactive exopolysaccharide was synthesised. Furthermore the incorporation of radioactivity into CM and polymer was sensitive to bacitracin suggesting the involvement of similar intermediates in X. campestris.

Preliminary experiments have indicated the presence of three anionic lipids (designated II, III and IV) in washed cells. On the basis of elution properties from DEAE-cellulose, resistance to mild alkali, relative rates of hydrolysis in mild acid, and susceptibility to phenol, together with analysis of released sugars, these lipids were tentatively identified as lipid monophosphate galactose (II), lipid diphosphate glucose (III) and lipid diphosphate cellobiose (IV). The nature of the lipid has not been established directly but the presence of isoprenoid alcohol and isoprenoid alcohol phosphate was indicated by tlc analysis of non-saponifiable lipids. No mass spectroscopy data is available.

Lipids with similar elution properties from DEAE cellulose were extracted from membrane preparations following incubation with UDP-[^{14}C]-glucose. The water-soluble polymeric products of particulate glucosyl transferase activity contained only glucose, apparently linked in a β-configuration. The in vitro synthesis of a polymer essentially similar to bacterial cellulose suggests analogies with A. xylinum, a bacterium producing cellulose in vivo. Reports concerning A. xylinum demonstrated the role of UDP-glucose as glucosyl donor (Colvin, 1959 ; Khan et al., 1961). Subsequent studies established the role of lipids tentatively identified as isoprenoid phosphates (Romero et al., 1977) as carriers of pyrophosphate linked glucose and cellobiose residues (Garcio et al., 1974).

The synthesis of polymer and CM material by Xanthomonas in vitro preparations was affected by the addition of UMP, UDP, glucose-1--phosphate and glucose suggesting the following reaction series:-

$$\text{UDP-glucose} + \text{lipid-P} \xrightleftharpoons{\text{glucose-1-P transfer}} \text{lipid-P-P-glucose} + \text{UMP}$$

$$\text{lipid-P-P-glucose} + \text{UDP-glucose} \xrightleftharpoons{\text{glucose transfer}} \text{lipid-P-P-cellobiose} + \text{UDP}$$

The in vitro synthesis of CM material was bacitracin sensitive. Addition of authentic C_{55}-P stimulated the incorporation of radioactivity into CM extracts and, to a lesser extent, polymer thus suggesting that the concentration of available lipid-P was limiting in cell-free systems.

No intermediates of chain length greater than disaccharide were extracted. It is unlikely that this observation was a reflection of the extraction technique since Hemming (1973) has used the established CMW technique (Behrens, Parodi and Leloir, 1971) to extract polyprenol phosphate residues carrying oligosaccharides comprising up to 20 glycosyl residues.

The role of lipid monophosphate galactose remains unclear. In washed cell suspension, turnover of galactose containing lipopolysaccharide occurs, thus a role can be postulated for the galactosyl lipid. The presence of a monophosphate linkage is however difficult to explain unless one postulates a system similar to that described for the modification of Salmonella O-antigen which involves isoprenoid monophosphate sugars (Wright, 1971). The presence of the galactosyl intermediate in a cell-free preparation catalysing synthesis of a glucosyl polymer cannot, however be explained in terms of a functional role.

Johnson et al., (1977) have isolated an intermediate characterised as lipid monophosphate glucose in a study of E. coli colanic acid synthesis. Synthesis of this lipid was unexpectedly inhibited by bacitracin; the authors suggest that this observation was an artifact. It is possible that the lipid moiety of the glucosyl lipid isolated differed in its structural configuration, from the other C_{55}--isoprenols detected in this membrane system. More recently (Yamamori, Murazumi, Araki and Ito, 1978), a membrane system from B. cereus strain

AHU 1356 was shown to synthesise three lipids. One lipid identified as C_{55}-diphosphate N-acetylglucosamine was sensitive to tunicamycin and was suggested to function in cell wall synthesis. Two further lipids were both characterised as C_{55}-monophosphate N-acetyl-glucosamine, but possibly containing lipid moieties differing in their geometrical structure.

The presence of the galactosyl lipid in X. campestris highlights the problem of the interconversion of supplied isotopically labelled UDP-glucose. In order to overcome this problem in E. aerogenes, Troy et al., (1971) used a strain defective in UDP-galactose-4-epimerase, unfortunately no similar strain was isolated in X. campestris.

The result of glucosyl transferase activity with the involvement of lipid diphosphate glucose and cellobiose derivatives in X. campestris, was a product comprising a mixture of β-linked glucosyl oligosaccharides. The production of a range of oligosaccharides is difficult to explain. Significant amounts of [^{14}C]-glucose may arise from several sources, one example being the hydrolysis of UDP[^{14}C]--glucose by sugar nucleotide hydrolases (Ward et al., 1968 ; 1969 ; Beacham et al., 1973), to release glucose and UDP. Alternatively, the cleavage of lipid intermediates would release both glucose and cellobiose with lipid diphosphate: the disaccharide may be subsequently hydrolysed by β-glucosidase activity. Although the β-glucosidase activity was confirmed in X. campestris there is no evidence to support hydrolysis of the lipid intermediates.

The occurrence of a β-glucosyl polymer in in vitro preparations was an unexpected result since analysis of X. campestris strains in vivo, did not reveal any polymer which could account for the in vitro product. It is however likely that the β-glucosyl chains may provide the 'backbone' of xanthan exopolysaccharide, to which side-chains could be attached either during active synthesis of the backbone, or alternatively and more likely, to preformed glucosyl oligosaccharide.

The apparent lack of mannosyl or glucuronosyl transferases from these particulate preparations is difficult to explain. No other mannosyl donors eg UDP-mannose or CDP-mannose were effective, this would be expected since only GDP-mannose was identified in the

cellular nucleotide pool. Tanner (1969) has described a system which synthesises yeast mannan, the system has a requirement for both Mn^{++} and Mg^{++}. Formation of prenyl phosphate mannosyl residues required Mg^{++} and the subsequent transfer of mannosyl residues was dependent upon the presence of Mn^{++}. Although X. campestris glucosyl trans--ferase showed an absolute requirement for Mg^{++}, addition of Mn^{++}, Zn^{++}, Ca^{++}, Co^{++} or Mo^{++} did not stimulate mannosyl transfer to either polymer or CM material. It is therefore unlikely that an overlooked ion requirement could account for the lack of activity. Attempts to demonstrate mannosyl transferase activity in soluble extracts also failed.

The violent preparative techniques (ie sonication) used in membrane preparation may disrupt membrane integrity or orientation, which would influence spatial separation of substrates and products or perhaps even enzyme and substrate.

Preparation of membranes by french-press or osmotic lysis did not restore mannosyl transferase activity, indeed sphaeroplasts prepared from X. campestris T646 have been used to synthesise a water-soluble polymer from [^{14}C]D-glucose; the polymer contained glucose but no mannose (R E Cripps, personal communication). Toluene-treated 'permeabilised' cells of X. campestris produced small quantities of polyglucose polymer from UDP[^{14}C]-glucose but no mannose was incorporated from GDP-mannose.

Attempts to improve membrane permeability by addition of detergents (eg. sodium sarkosinate, Triton X-100 and Span 20) failed; no mannosyl transfer occurred and glucosyl transfer was reduced or inhibited.

It is perhaps significant that parallel preparations of E. aerogenes membranes produced an in vitro system capable of incorporating glucose, galactose and glucuronic acid, (ie all the known component sugars for this particular polymer/into polymer (C L Saunderson, personal communication).

Addition of initiators or chain acceptors is important in several bacterial polysaccharide synthesising systems. The roles of cellodextrins and maltodextrins in the synthesis of bacterial cellulose (Cooper et al., 1975b) and glycogen (Barengo, Flawia and

Krisman, 1975) are well documented. Addition of short chains comprising 8-12 repeating units has been shown to stimulate synthesis of Pneumococcal type III polymer (Smith and Mills, 1962).

Addition of presynthesised, unlabelled polyglucose chains ie cellodextrins from A. xylinum or X. campestris T646 or 646E in vitro incubations stimulated glucosyl transfer and chain elongation in X. campestris. Preincubation with unlabelled UDP-glucose had a similar effect. Use of mucoid bacteria for the preparation of membranes might suggest that endogenous polysaccharide material occurred within those membranes.

Glycoproteins with attached cellodextrins serve as primers for cellulose synthesis from UDP-glucose in A. xylinum (Franz, 1976). Independent reports have demonstrated the synthesis of alkali soluble $\beta 1 \rightarrow 4$ glucans which were subsequently converted into alkali insoluble glucans, in encysting cells of Acanthamoeba castellanii (Neff) (Potter and Wiseman, 1971; Stewart and Weisman, 1974 ; Potter and Wiseman, 1976) and also in membranes from peas (Brett and Northcote, 1975).

An unusual glucosyl polymer has also been reported in A. xylinum (Colvin, Chéné, Sowden and Takai, 1977) ; the oligosaccharide was heterogeneous in size and consisted of a linear $\beta 1 \rightarrow 4$ linked polymer with single glucose residues at the 2 position of every third glucose in the chain (on average). The authors have suggested that this soluble material may perhaps function as an intermediate in the synthesis of bacterial cellulose. By removal of side chains from the oligosaccharide, the polymer would become insoluble forming micro--fibrils, but its solubility in the presence of side chains would ease transport into the environment.

Addition of cellodextrins to Xanthomonas T646 membranes also resulted in low, but significant, levels of mannosyl transfer from GDP-mannose to polymer. It is possible that the glucosyl oligosaccha--rides are required as intermediates or primers in the biosynthesis of xanthan by X. campestris.

On the basis of the results presented here, the synthesis of exopolysaccharide by X. campestris appears to proceed by a mechanism differing from that described for E. aerogenes (Sutherland et al.,

1970 ; Troy et al., 1971). Synthesis of β1→4 linked polyglucose backbone involves synthesis of a lipid-linked disaccharide, followed by transfer to cellodextrins. The apparent absence of mannose or glucuronic acid containing lipid intermediates in washed cell CM extracts under conditions where mannose containing exopolysaccharide was synthesised, points to a significant difference in X. campestris exopolymer biosynthesis. No radioactivity was incorporated into CM extracts from GDP[^{14}C]-mannose by in vitro preparations. It would therefore appear that transfer of mannose at least, did not involve an isoprenoid lipid and thus would not be expected to be bacitracin sensitive. Addition of mannosyl residues occurred to presynthesised glucosyl oligo--saccharides.

A similar system has been shown to operate in synthesis of E. coli 08 and 09 antigens and certain Klebsiella O-antigens (Fleming et al., 1978 ; Kopmann et al., 1975). In vitro synthesis the mannan O-antigen required a butanol soluble glucolipid (Kanegasaki and Jann, 1979) to which mannosyl residues were transferred directly from GDP-mannose without the involvement of an isoprenoid lipid.

Transfer of glycosyl residues from sugar nucleotides directly to polysaccharide without polyprenol intermediates, has been widely reported in eukaryotic systems. Staneloni and Lelior (1979) have recently reviewed the synthesis of aspargine-linked oligosaccharides involved in mammalian glycoprotein assembly. An oligosaccharide containing 7-9 glycose units, comprising α-and β-mannosyl units with two N-acetylglucosamine residues at the reducing terminus (Hsu, Baynes and Heath, 1974) and therefore resembling the core structure of many glycoproteins, was synthesised on a dolichol diphosphate molecule. Following release of the oligosaccharide to a polypeptide acceptor, the nascent glycoprotein was modified by removal of specific monosaccharides or by addition of others. This addition occurs directly from sugar nucleotide without dolichol involvement and results in the release of nucleotide diphosphate. A mannosyl trans--ferase capable of catalysing the transfer of mannose from GDP-mannose directly to complex peptidophosphogalactomannan without the involve-

-ment of isoprenoid lipids, has been solubilised from Penicillium charlesii membranes using Triton X-100 (Gander and Fang, 1977).

One possible mechanism for exopolysaccharide biosynthesis in X. campestris, (fig 60) would initially require the transfer of lipid-linked disaccharides to a specific receptor site (possibly a specific protein) at the outer surface of the cytoplasmic membrane. If the specific receptor site is localised, then localisation of carrier lipid within the cytoplasmic membrane would be necessary. Reports concerning lipopolysaccharide biosynthesis have suggested localised sites where de novo synthesised material can enter the existing lipopolysaccharide layer (Kulpa et al., 1976), this system might also suggest localisation of carrier lipid.

If the sites of mannosyl and glucuronosyl transferases were adjacent to the glucosyl receptor site, allowing interaction with the nascent glucosyl chain, addition of side chains to the disaccharide could occur concomittantly with disaccharide addition. This system would be dependent upon periplasmic GDP-mannose UDP-glucuronic acid, since without the action of carrier lipid, these hydrophilic precursors could not cross the hydrophobic cytoplasmic membrane. The discovery of periplasmic 5′-nucleotidases in E. coli (Beacham et al., 1973) suggests that in this organism at least, sugar nucleotides occur in the periplasm ; no data was presented concerning the range and concentration of periplasmic nucleotides. The enzyme phosphoglucose isomerase, involved in the synthesis of fructose-6-phosphate and hence possibly GDP-mannose from glucose-6-phosphate (see fig. 26) is located in part at least in the periplasm of E. coli (Friedberg, 1972) and S. typhimurium (Chatterjee et al, 1976). As a result, this enzyme is often released from 'leaky' mutants.

It is possible however that mannosyl and glucuronosyl transferases are situated at the outer membrane and act as the glucosyl chain passes through the outer membrane.

Discrete pores for the export of exopolysaccharide have been reported in E. coli (Bayer et al., 1977) and A. xylinum (Zaar, 1978). Whether these pores are stationary or capable of moving over the cell

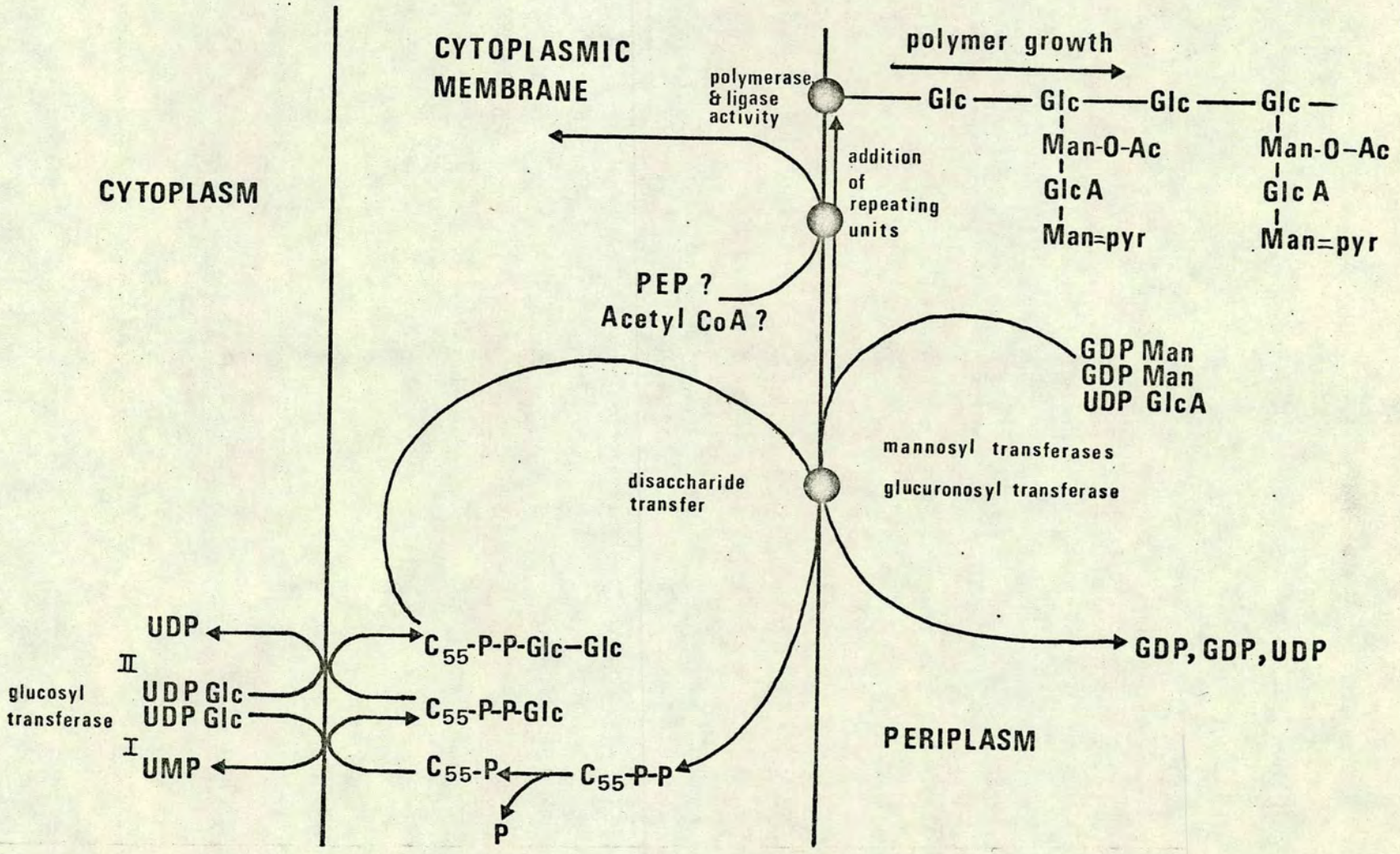


Fig. 60 Proposed model for the Biosynthesis of exopolysaccharide by X. campestris.

surface, has not been established. With a fixed receptor site at the cytoplasmic membrane, localisation of the export site might be expected.

Ligase activity at the cytoplasmic membrane would release free slime exopolysaccharide. Although a product of uniform molecular weight is usually released (see Table 3), the mechanism for chain length determination is, as yet, unknown.

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